

## PATENT APPLICATION

### **METHODS AND COMPOSITIONS FOR EXPRESSING POLYNUCLEOTIDES SPECIFICALLY IN SMOOTH MUSCLE CELLS IN VIVO**

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## **METHODS AND COMPOSITIONS FOR EXPRESSING POLYNUCLEOTIDES SPECIFICALLY IN SMOOTH MUSCLE CELLS IN VIVO**

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### CROSS-REFERENCES TO RELATED APPLICATIONS

The present application claims priority to U.S. Provisional Application No.

60/263,811, filed January 24, 2001. The present application is also a continuation-in-part application of U.S. Patent Application No. 09/600,319, filed July 13, 2000, which is a U.S. national

10 phase application of international application number PCT/US99/01038, filed January 15, 1999, which claims priority to U.S. Provisional Application No. 60/071,300, filed January 16, 1998. The invention is also related to international application PCT/US99/24972. Each of the aforementioned applications is explicitly incorporated herein by reference in its entirety and for all purposes.

### STATEMENT AS TO FEDERAL FUNDING

Work described herein has been supported in part by National Institutes of Health grants R01 HL38854 and P01 HL19242. The U. S. Government may therefore have certain rights in the invention.

### FIELD OF THE INVENTION

This invention relates generally to the field of regulation of gene expression, and specifically to smooth muscle specific promoters and enhancers. The invention also relates to methods of modulating gene expression by utilizing smooth muscle specific promoters and enhancers.

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### BACKGROUND OF THE INVENTION

Smooth muscle cells (SMCs), often termed the most primitive type of muscle cell because they most resemble non-muscle cells, are called "smooth" because they contain no striations, unlike skeletal and cardiac muscle cells. Smooth muscle cells aggregate to form smooth muscle which constitutes the contractile portion of the stomach, intestine and uterus, the walls of

arteries, the ducts of secretory glands and many other regions in which slow and sustained contractions are needed.

Abnormal gene expression in SMC plays a major role in numerous diseases including, but not limited to, atherosclerosis, hypertension, stroke, asthma and multiple 5 gastrointestinal, urogenital and reproductive disorders. These diseases are the leading causes of morbidity and mortality in Western Societies, and account for billions of dollars in health care costs in the United States alone each year.

In recent years, the understanding of muscle differentiation has been enhanced greatly with the identification of several key *cis*-elements and *trans*-factors that regulate expression 10 of muscle-specific genes. Firulli A. B., *et al.*, 1997, *Trends in Genetics*, 13:364-369; Sartorelli V. *et al.*, 1993, *Circ. Res.*, 72:925-931. However, the elucidation of transcriptional pathways that govern muscle differentiation has been restricted primarily to skeletal and cardiac muscle.

Currently, no transcription factors have yet been identified that direct smooth muscle-specific gene expression, or SMC myogenesis. Owens G.K., 1995, *Physiol. Rev.*, 75:487-517. Unlike skeletal 15 and cardiac myocytes, SMC do not undergo terminal differentiation. Furthermore, they exhibit a high degree of phenotype plasticity, both in culture and *in vivo*. Owens G.K., 1995, *Physiol. Rev.*, 75:487-517; Schwartz, S.M. *et al.*, 1990, *Physiol. Rev.*, 70:1177-1209. Phenotype plasticity is particularly striking when SMC located in the media of normal vessels are compared to SMC located in intimal lesions resulting from vascular injury or atherosclerotic disease. Schwartz, S.M.

20 *et al.*, 1990, *Physiol. Rev.*, 70:1177-1209; Ross R., 1993, *Nature*, 362:801-809; Kocher O. *et al.*, 1991, *Lab. Invest.*, 65:459-470; Kocher O. *et al.*, 1986, *Hum. Pathol.*, 17:875-880. Major modifications include decreased expression of smooth muscle isoforms of contractile proteins, altered growth regulatory properties, increased matrix production, abnormal lipid metabolism and decreased contractility. Owens G.K., 1995, *Physiol. Rev.*, 75:487-517. The process by which SMC

25 undergo such changes is referred to as "phenotypic modulation". Chamley-Campbell J.H. *et al.*, 1981, *Atherosclerosis*, 40:347-357. Importantly, these alterations in expression patterns of SMC protein cannot simply be viewed as a consequence of vascular disease, but rather are likely to contribute to the progression of the disease.

Expression of smooth muscle myosin heavy chain (SM-MHC) appears to be 30 completely restricted to SMC lineages throughout development (Miano J. *et al.*, 1994, *Circ. Res.*, 75:803-812). To date, four SM-MHC isoforms (SMC-1A, SMC-1B, SMC-2A, and SMC-2B) have been identified (Nagai R. *et al.*, 1989, *J. Biol. Chem.*, 264:9734-9737; White S. *et al.*, 1993, *Am. J. Physiol.*, 264:C1252-C1258; Kelley C.A. *et al.*, 1993, *J. Biol. Chem.*, 268:12848-12854), all of

which are derived from alternative splicing of a single gene (Miano J. *et al.*, 1994, *Circ. Res.*, 75:803-812; Babij P. *et al.*, 1989, *J. Mol. Biol.*, 210:673-679). Alterations in expression of SM-MHC isoforms have been extensively documented in SMC that have undergone phenotypic modulation either when placed in culture (Rovner A.S., 1986, *J. Biol. Chem.*, 261:14740-14745; 5 Kawamoto S. *et al.*, 1987, *J. Biol. Chem.*, 262:7282-7288), or in vascular lesions of both humans and several animal models of vascular disease (Aikawa M. *et al.*, 1997, *Circulation*, 96:82-90; Sartore S. *et al.*, 1994, *J. Vasc. Res.*, 31:61-81).

Transcriptional regulation of the SM-MHC gene has been analyzed in cultured SMC and several functional *cis*-elements have been identified. White S.L. *et al.*, 1996, *J. Biol. Chem.*, 10 271:15008-15017; Katoh Y. *et al.*, 1994, *J. Biol. Chem.*, 269:30538-30545; Watanabe M. *et al.*,

1996, *Circ. Res.*, 78:978-989; Kallmeier R.C. *et al.*, 1995, *J. Biol. Chem.*, 270:30949-30957; Madsen C.S. *et al.*, 1997, *J. Biol. Chem.*, 272:6332-6340; Madsen C.S. *et al.*, 1997, *J. Biol. Chem.*, 15 272:29842-29851. However, because differentiation of SMC is known to be dependent on many local environmental cues that cannot be completely reproduced in vitro, cultured SMC are known to be phenotypically modified as compared to their *in vivo* counterparts (Owens G.K., 1995, *Physiol. Rev.*, 75:487-517; Chamley-Campbell J.H. *et al.*, 1981, *Atherosclerosis*, 40:347-357). As such, certain limitations may apply regarding the usefulness of cultured SMC in defining transcriptional programs that occur during normal SMC differentiation and maturation within the animal.

20 A few promoters relating to smooth muscles have been described in the art, e.g., promoters for SM-actin and SM22 genes. However, a major disadvantage with these promoters is that they are clearly not SMC specific. SM22 and SM-actin are highly expressed in myofibroblasts during wound repair, within granulomatous tissues, tumors, etc. The promoters for these genes are also transiently activated in skeletal and cardiac muscle during development, and in association 25 with a number of pathological circumstances (e.g. myocardial hypertrophy). In addition, the SM22 promoter fragments tested to date also have very little activity in SMC tissues of adult mice. Thus, such promoters have major limitations in terms of their utility in smooth muscle tissue specific targeting and expression *in vivo*.

Thus, there is a need in the art for transcription regulatory sequences (e.g., promoters 30 and enhancers) that can direct gene expression specifically in smooth muscle tissues *in vivo* (e.g., in human or non-human animals). There is also a need for relatively small smooth muscle specific promoter/enhancers that retain high level SMC specific expression *in vivo* and yet are selectively active in subsets of SMC (e.g. vascular versus gastrointestinal SMC, large versus small arteries,

pulmonary versus gastrointestinal SMC, etc.). Methods for utilizing such SMC specific promoters and enhancers to target delivery and expression of polynucleotide to SMCs are also needed. The present invention fulfills these and other needs.

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#### SUMMARY OF THE INVENTION

In one aspect, the present invention provides isolated or recombinant polynucleotides which comprise a smooth muscle myosin heavy chain (SM-MHC) promoter/enhancer sequence capable of conferring smooth muscle specific expression *in vivo*. In some of the polynucleotides, the promoter sequence consists essentially of a sequence selected 10 from (i) the region of nucleotides 5663 to 5889 of SEQ ID NO:16; (ii) SEQ ID NO:16 except that CArG2 has been mutated; (iii) SEQ ID NO:16 except that the intronic CArG has been mutated; (iv) the regions of nucleotides 1 to 6,700 and nucleotides 9,500 to 15,800 of SEQ ID NO:16; (v) the regions of nucleotides 1 to 9,500 and nucleotides 11,700 to 13,700 of SEQ ID NO:16; (vi) SEQ ID NO:16; and (vii) SEQ ID NO:17.

15 Some of the polynucleotides hybridize under stringent conditions to the SM-MHC promoter/enhancer. Some of the polynucleotides further comprise a heterologous polynucleotide operably linked to the SM-MHC promoter sequence. Some of the heterologous polynucleotides encode a polypeptide. The polypeptide can be a toxin, a prodrug-converting enzyme, a tumor suppressor, a sensitizing agent, an apoptotic factor, an angiogenesis inhibitor, a cytokine, or an 20 immunogenic antigen. Some of the heterologous polynucleotides consist of an antisense polynucleotide or a catalytic polynucleotide.

In another aspect, the invention provides expression vectors which comprise a smooth muscle myosin heavy chain (SM-MHC) promoter/enhancer sequence that confers smooth muscle specific expression *in vivo*. Some of the expression vectors are retroviral vectors, adenovirus-associated viral vectors, or adenoviral vectors. Some of the expression vectors have the promoter sequence operably linked to a heterologous polynucleotide. Some of the expression vectors comprise a promoter which consists essentially of the sequence of SEQ ID NO:16 except that CArG2 or the intronic CArG has been mutated.

25 In another aspect, the invention provides genetically engineered host cells comprising an expression vector of the invention. Transgenic non-human animals containing the polynucleotides of the invention are also provided. The invention also provides pharmaceutical compositions which comprise the polynucleotides of the invention in a pharmaceutically acceptable carrier.

In still another aspect, the present invention provides methods of expression a polynucleotide in a smooth muscle cell *in vivo*. The methods entail introducing into the smooth muscle cell the polynucleotide that is operably linked to an SM-MHC promoter/enhancer sequence capable of conferring smooth muscle specific expression *in vivo*. In some of the methods, the 5 promoter/enhancer consists essentially of (i) the region of nucleotides 5663 to 5889 of SEQ ID NO:16; (ii) SEQ ID NO:16 except that CArG2 has been mutated; (iii) SEQ ID NO:16 except that the intronic CArG has been mutated; (iv) the regions of nucleotides 1 to 6,700 and nucleotides 9,500 to 15,800 of SEQ ID NO:16; (v) the regions of nucleotides 1 to 9,500 and nucleotides 11,700 to 13,700 of SEQ ID NO:16; (vi) SEQ ID NO:16; or (vii) SEQ ID NO:17.

10 In some methods, the polynucleotide to be expressed is a reporter gene. In some other methods, the polynucleotide to be expressed encodes a therapeutic protein. In some methods, the SM-MHC promoter/enhancer enables expression of the polynucleotide specifically in coronary artery, aorta, airway smooth muscle, or pulmonary vascular smooth muscle. In some methods, the 15 SM-MHC promoter/enhancer enables expression of the polynucleotide specifically in bladder smooth muscle, gastrointestinal tract smooth muscle, or urinary tract smooth muscle. In some other methods, the SM-MHC promoter/enhancer enables expression of the polynucleotide specifically in aorta, pulmonary airway, or pulmonary vascular smooth muscle. In still some other methods, the SM-MHC promoter/enhancer enables expression of the polynucleotide specifically in 20 gastrointestinal tract smooth muscle, urinary tract smooth muscle, airway smooth muscle, vein smooth muscle, or small branching artery smooth muscle. In some methods, the SM-MHC promoter/enhancer enables expression of the polynucleotide specifically in aorta artery smooth muscle, carotid artery smooth muscle, pulmonary artery smooth muscle, vena cava vein smooth muscle, or vascular smooth muscle.

25 In yet another respect, the invention provides methods for screening compounds that modulate the activity of an SM-MHC promoter/enhancer. The methods entail contacting a test compound with a cell that contains the SM-MHC promoter/enhancer operably linked to a reporter gene; detecting expression of the reporter gene; and comparing the expression thus detected with the amount of expression obtained in the absence of the test compound. If the level obtained in the presence of the test compound is higher or lower than that obtained in the absence of the test 30 compound, a compound that modulates the activity of the SM-MHC promoter/enhancer has been identified.

A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification, the figures and claims.

All publications, GenBank deposited sequences, ATCC deposits, patents and patent applications cited herein are hereby expressly incorporated by reference in their entirety and for all purposes.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows expression of the rat SM-MHC -4.2 to +11.6 promoter-lacZ gene in vivo in adult transgenic mice showing the SMC specificity of the promoter. Extremely high expression was observed in virtually all SMC tissues with no expression in non-SMC (Figure 3).

10 Figure 2 shows analysis of the SMC specificity of the rat SM-MHC promoter in various SMC tissues of transgenic mice in vivo using a cre recombinase indicator system.

Transgenic mice carrying a SM MHC-cre recombinase gene were crossed to an indicator line containing a lox p (the cre recognition site) flanked stop codon inserted upstream of a lacZ reporter gene that was inserted into the ubiquitously expressed ROSA gene locus by homologous recombination (the mouse is designated R26R). Results showed expression of the lacZ indicator

15 gene in virtually all SMC tissues. These results thus provide extremely compelling evidence for the SMC specificity of the -4.2 to +11.6 SM MHC promoter, since this assay system is far more sensitive in detecting reporter expression than conventional direct reporter systems in that even transient activation of the promoter is detected. That is, once cre recombination occurs there is permanent activation of the lacZ reporter. These results also establish the feasibility of using the

20 SM MHC promoter in conjunction with cre recombinase and conditional (e.g. tetracycline etc.) gene regulatory systems for purposes of achieving SMC specific gene targeting that is regulatable.

A1-Thoracic aorta from a SMMHC-cre X R26R mouse; B-Trachea and carotid arteries; C-Heart; D-Lung; E-Skeletal muscle arteriole; F-Mesenteric vessels; G-Ventral surface of cerebrum; H1-Jejunum from a SMMHC-cre X R26R mouse; I-Bladder. The results provide rigorous assessment

25 of the complete SMC specificity of the SM MHC promoter.

Figure 3 shows histological assessment of LacZ expression in the SMC tissues shown in Fig. 2 showing the remarkable SMC specificity of expression of the -4.2 to +11.6 SM-MHC promoter. The results showed complete specificity of expression of LacZ within SMC with the exception of a very small population of atrial myocytes that show transient activation of the 30 promoter during early heart formation (see reference {6812} f). A1- cross section of carotid artery; A2-cross section of aorta; B1- cross section of intramyocardial artery; B2- staining of a small population of cardiac myocytes; C-cross section of trachea; D- cross section of lung showing both

bronchiole and pulmonary artery staining; E- skeletal muscle arteriole and venule; F- cross section of jejunum; G- cross section of esophagus; H-cross section of ureter.

Figure 4 shows expression of the rat SM-MHC -4.2 to +5.3/+7.5 to +9 promoter LacZ gene in various tissues from adult transgenic mice. As seen, high reporter expression was seen in multiple SMC tissues including the coronary arteries, aorta, airway SMC, and pulmonary vascular SMC (PA-pulmonary artery). The results indicate that this derivative of the SM MHC promoter retains high activity in aortic SMC, pulmonary arterial SMC, and airway SMC.

Figure 5 shows histological section of the -4.2 to +5.3/+7.5 to +9 SM-MHC promoter showing high specificity of expression in pulmonary arteries and arterioles (see arrow).

Figure 6 shows expression of the -4.2 to +2.5 and +5.3 to +11.6 SM-MHC LacZ gene in tissues of adult transgenic mice. As seen, this SM MHC promoter reporter construct retained high level expression in the pulmonary airways, and aorta but diminished expression in the coronary arteries as compared to the wildtype -4.2 to +11.6 SM MHC LacZ transgene construct (see Figures 1-3). There was also high level expression in pulmonary vascular SMC based on histological analyses (data not shown). The results indicate high activity in pulmonary artery SMC, airway SMC, and the aorta, but virtually no activity in coronary artery SMC.

Figure 7 shows transgene expression of the intronic CArG region-minimal TK-LacZ. Various tissues of 4-week-old transgenic mice and embryos of the 3xICR-TK LacZ line (7240) were stained for  $\beta$ -galactosidase activity. A-B: anterior view of the heart and lung; C: the esophagus, stomach, and duodenum; D: a part of small intestine; E: the bladder; F: bottom view of the brain; G: anterior view of abdominal organs and great blood vessels; H-K: histological examination of the thoracic aorta (H), pulmonary artery and bronchus (I), cardiac muscle and coronary artery (J), and intercostal muscle (K) of the 3xICR-TK LacZ transgenic mice; L-M: transgene expression in a 19.5-dpc embryo of the 3xICR-TK LacZ line. The embryo was skinned, sectioned sagittally along the midline, stained, and cleared; N: transgene expression in the heart and aorta of a 16.5-dpc embryo. Ao indicates aorta; PA, pulmonary artery; SMA, superior mesenteric artery; IVC, inferior vena cava; H, heart; Br, bronchus; Eso, esophagus; Int, intestine; S, stomach; Bl, bladder. The results indicate that a very small derivative of the promoter is capable of driving high level expression in SMC *in vivo*.

Figure 8 shows the effects of mutation of the intronic CArG on expression of the rat -4.2 to +11.6 SM-MHC transgene *in vivo*. Abdominal organs were removed en block showing reporter expression in the blood vessels and urinary tract in the wild-type (A) and intronic CArG mutant (B) transgenic mice. To better illustrate transgene expression in large arteries, several

smaller arteries and connective tissues were removed and the tissues cleared. The supramesenteric artery, which was stained positive, was removed from the intronic CArG mutant mouse tissues. A portion of the tissues is expanded in the insert in panel B. Arrowheads indicate the position of aorta that is not visible because of the lack of staining. Note that the blood vessels within the kidneys 5 were not stained in either wild-type nor intronic CArG mutants. C, D, the thoracic aorta and branching arteries of the wild-type (C) and the intronic CArG (D) mutant transgenic mice. E, view of the large arteries in the cervicothoracic region of the intronic CArG mutant transgenic mouse. F, the large arteries and their branches in the abdomen of the intronic CArG mutant. A portion of the arteries is expanded in the insert in F. G, H, I, J, histological examination of the abdominal aorta 10 and inferior vena cava of the wild-type (G, I) and the intronic CArG mutant (H, J) mice showing abrogation of reporter expression in SMCs of the aorta in the intronic CArG mutants. Note that expression in the vena cava was not changed by the mutation. The boxed areas (G, H) are shown 15 by a higher magnification (I, J). Ao indicates aorta; DA, ductus arteriosus; IVC, inferior vena cava; SCA, subclavian artery. The results indicate that this mutation selectively abolished activity in large blood vessels such as the aorta, carotid, and coronary outflow tracts without altering expression in smaller arteries and arterioles.

Figure 9 shows activity of human SM-MHC promoter of -5.1 to + 13.5 region in transgenic mice. Expression of the human MHC-5.1/13.5-LacZ transgene in adult (5-6 weeks old) mouse tissues. Whole tissues were processed and stained for lacZ expression as previously 20 described (Madsen et al. *Circ. Res.* 82:908-917, 1998). Results show that the human promoter has activity virtually identical to that of the rat SM-MHC promoter.

Figure 10 shows histological evaluation of human MHC -5.1/13 in transgenic mice. Histological examination of specificity of expression of the human MHC-5.1/13.5-LacZ transgene in adult (5-6 weeks old) mouse tissues. Tissues were processed and stained for lacZ expression as 25 previously described (Madsen et al. *Circ. Res.* 82:908-917, 1998).

Figure 11 shows nucleotide sequence comparison of the rat and human SM-MHC promoter/enhancer sequence within the 5' promoter region. As indicated, there is complete sequence homology between the rat and human genes in the key regulatory regions identified thus far (e.g. 5' CArG 1, 2 and 3; the G/C repressor, etc., as indicated). The identity of these elements in 30 the rabbit and mouse genes have been shown previously. See, Iadsen et al., 1997, *J. Biol. Chem.*, 272:6332.

Figure 12 shows gross examination of SM-MHC 4.2-Intron-lacZ expression in various smooth muscle containing tissues. Transgenic mice (5-6 week-old) were perfusion fixed

with a 2% formaldehyde/0.2% paraformaldehyde solution and various smooth muscle containing tissues were harvested and stained overnight at room temperature for  $\beta$ -galactosidase activity using 5-bromo-chloro-3-indolyl- $\beta$ -D galactopyranoside (X-Gal) as the substrate.

Figure 13 shows histological analysis of rat SM-MHC 4.2-Intron-*lacZ* expression in various smooth muscle containing tissues. Transgenic mice (5-6 week-old) were perfusion fixed with a 2% formaldehyde/0.2% paraformaldehyde solution and various smooth muscle containing tissues were harvested and stained overnight at room temperature for  $\beta$ -galactosidase activity using 5-bromo-chloro-3-indolyl- $\beta$ -D galactopyranoside (X-Gal) as the substrate. After staining with X-Gal overnight, tissues were processed for paraffin embedding, sectioned at 6 $\mu$ m, and sections counterstained with hematoxylin/eosin.

Figure 14 shows expression of SM-MHC 4.2-Intron-*lacZ* throughout development.

Embryos were harvested at various time points (10.5 - 16.5 days p.c.), fixed with a 2% formaldehyde/0.2% paraformaldehyde solution and stained overnight at room temperature for  $\beta$ -galactosidase activity using 5-bromo-chloro-3-indolyl- $\beta$ -D galactopyranoside (X-Gal) as the substrate. Embryos were then cleared in benzyl benzoate:benzyl alcohol (2:1). Panel A: 10.5 days p.c. Panel B: 12.5 days p.c. Panel C: 14.5 days p.c. Panel D: 16.5 days p.c.

Figure 15 shows expression of SM-MHC 4.2-Intron-*lacZ* at 19.5 days p.c. Embryos were harvested at 19.5 days p.c., fixed with a 2% formaldehyde/0.2% paraformaldehyde solution and stained overnight at room temperature for  $\beta$ -galactosidase activity using 5-bromo-chloro-3-indolyl- $\beta$ -D galactopyranoside (X-Gal) as the substrate. Embryos were then cleared in benzyl benzoate:benzyl alcohol (2:1). Panel A: Sagittal section of 19.5 day embryo. Panel B: Closeup of thoracic cavity. Panel C: Iliac artery and vein. Panel D: Vessels within the musculature of the thoracic wall.

Figure 16 shows expression of the SM-MHC 4.2-Intron-*lacZ* transgene in the coronary circulation of the heart of an adult mouse. High levels of SMC-specific expression are present in all major coronary arteries and arterioles.

Figure 17 shows schematic representation of the rat SM-MHC 4.2-Intron-*lacZ* clone and a comparable region of the human SM-MHC gene. As indicated, there is conservation of key regulatory elements including the CArG boxes, the GC repressor and an NF-1 site.

Figures 18A and 18B show mutants with deletions in the intronic CArG element and their promoter activity. (A) A series of 3'-end deletion mutants of the SM-MHC *LacZ* sequence was generated and assayed for reporter activity in cultured rat SMCs. The  $\beta$ -galactosidase activity of each construct is expressed relative to the activity of the promoterless pAUG *LacZ*. Error bars

show standard error. (B) The nucleotide sequence (+1535 to +1703 from the transcription start site) of a portion of the rat SM-MHC first intron (SEQ ID NO:16) was compared with the corresponding human genomic sequence (GenBank U91323)(SEQ ID NO:17). The intronic CArG element is boxed. Note that the human intronic CArG lacks a G-substitution within the central 5 A/T-rich sequence and perfectly match the CArG consensus (CC(A/T)<sub>6</sub>GG). *Bold letters* indicate the region used in 3xICR TK *LacZ* construct. Nucleotides conserved with the rat sequence are indicated by *dashes*. Nucleotide additions are indicated by *lower-case letters*.

Figure 19 show EMSA analysis of the CArG elements using tissue nuclear extracts.

Radiolabeled 20-bp of double stranded oligonucleotides encompassing CArG1, CArG2, intronic 10 CArG, and *c-fos* SRE were incubated with either nuclear extracts prepared from tissues or SMCs or recombinant serum response factor (SRF). The amount of nuclear extracts was determined to produce SRF shift bands of similar intensity: 4  $\mu$ g of aortic; 3  $\mu$ g of bladder; 3  $\mu$ g of stomach; 7  $\mu$ g 15 of heart; 3  $\mu$ g of liver; and 5  $\mu$ g of rat SMCs nuclear extracts. One  $\mu$ l of programmed lysate of *in vitro* transcription/translation system (Promega) was used for recombinant SRF.

Figure 20 shows macroscopic examination of reporter gene expression in wild-type and mutant SM-MHC *LacZ* transgenic mice. Four- to 6-week-old transgenic mice were perfusion-fixed with a 2% formaldehyde/0.2% glutaraldehyde solution. Pictures show *LacZ* reporter expression in various tissues from wild-type -4200/+11600 *LacZ* (A, E, I, M, Q), CArG1 mutant (B, F, J, N, R), CArG2 mutant (C, G, K, O, S), and intronic CArG mutant mice (D, H, L, P, T). A-20 D, anterior view of the heart and aorta. E-H, the lung. I-L, the esophagus, stomach, and duodenum. M-P, a portion of small intestine. Q-T, the bladder. Tissues were cleared by benzyl benzoate/benzyl alcohol in A-H.

Figure 21 shows large artery-specific silencing of the reporter gene in intronic CArG 25 mutant mice. Abdominal organs removed en block showing reporter expression in the blood vessels and urinary tract in the wild-type (A) and intronic CArG mutant (B) transgenic mice. To better illustrate transgene expression in large arteries, several smaller arteries and connective tissues were removed and the tissues cleared. The supramesenteric artery, which was stained positive, was removed from the intronic CArG mutant mouse tissues. A portion of the tissues is expanded in the 30 insert in panel B. Arrowheads indicate the position of aorta that is not visible because of the lack of staining. Note that the blood vessels within the kidneys were not stained in either wild-type nor intronic CArG mutants. C, D, the thoracic aorta and branching arteries of the wild-type (C) and the intronic CArG (D) mutant transgenic mice. E, view of the large arteries in the cervicothoracic region of the intronic CArG mutant transgenic mouse. F, the large arteries and their branches in the

abdomen of the intronic CArG mutant. A portion of the arteries is expanded in the insert in F. G, H, I, J, histological examination of the abdominal aorta and inferior vena cava of the wild-type (G, I) and the intronic CArG mutant (H, J) mice showing abrogation of reporter expression in SMCs of the aorta in the intronic CArG mutants. Note that expression in the vena cava was not changed by 5 the mutation. The boxed areas (G, H) are shown by a higher magnification (I, J). Ao indicates aorta; DA, ductus arteriosus; IVC, inferior vena cava; SCA, subclavian artery.

Figure 22 shows transgene expression in embryos. Embryos were harvested at 19.5 dpc, skinned, and sectioned sagittally along the midline to permit dye penetration. The embryos were stained and cleared. The staining seen on the intestines in the negative and CArG2 mutant 10 transgenic mice is due to endogenous  $\beta$ -galactosidase activity and limited within the epithelial layer. Ao indicates aorta; Eso, esophagus; H, heart; St, stomach; Tr, trachea.

Figure 23 shows supershift analysis of the intronic CArG-binding proteins. One  $\mu$ l of anti-SRF antibody was added to the binding reaction of an intronic CArG probe and nuclear extracts after 20 min of incubation on ice and the reactions were further incubated for 10 min on 15 ice. Addition of the antibody resulted in supershift of SRF-containing complexes (A, B). Complexes A and B formed with other CArG probes used in EMSAs in Fig. 23 were also supershifted (data not shown). Arrows indicate supershifted complexes.

Figure 24 shows chromatin immunoprecipitation analysis of SRF binding to the endogenous CArG regions. PCR was carried out to detect the endogenous CArG regions in 20 immunoprecipitated chromatin fragments. Lanes 1, 4, 7, 10 show PCR amplification of control precipitation samples with no antibody. Lanes 2, 5, 8, and 11 shows amplification of 1:100 dilution samples of total input DNA for immunoprecipitation. Lanes 3, 6, 9, and 12 show amplification of target sequences in immunoprecipitated chromatin fragments with anti-SRF antibody.

Figure 25 shows transgene expression of the intronic CArG region-minimal TK-  
25 LacZ. Various tissues of 4-week-old transgenic mice and embryos of the 3xICR-TK LacZ line (7240) were stained for  $\beta$ -galactosidase activity. A, B, anterior view of the heart and lung. C, the esophagus, stomach, and duodenum. D, a part of small intestine. E, the bladder. F, bottom view of the brain. G, anterior view of abdominal organs and great blood vessels. H-K histological examination of the thoracic aorta (H), pulmonary artery and bronchus (I), cardiac muscle and 30 coronary artery (J), and intercostal muscle (K) of the 3xICR-TK LacZ transgenic mice. L, M, transgene expression in a 19.5-dpc embryo of the 3xICR-TK LacZ line. The embryo was skinned, sectioned sagittally along the midline, stained, and cleared. N, transgene expression in the heart and aorta of a 16.5-dpc embryo. Ao indicates aorta; PA, pulmonary artery; SMA, superior

mesenteric artery; IVC, inferior vena cava; H, heart; Br, bronchus; Eso, esophagus; Int, intestine; S, stomach; Bl, bladder.

#### DETAILED DESCRIPTION OF THE INVENTION

##### 5 I. Overview

The invention provides novel isolated or recombinant polynucleotides comprising cis-acting transcriptional control sequences of smooth muscle (SM) myosin heavy chain (SM-MHC) genes that confer smooth muscle cell (SMC) specific gene expression both *in vitro* (e.g., in cultured cells) and *in vivo* (e.g., in human or transgenic animals). The invention also provides

10 polynucleotides and expression vectors comprising SMC specific transcription regulatory elements that are active in only certain subtypes of SM cells. The polynucleotides of the invention include those based on or derived from genomic sequences of untranscribed, transcribed and intronic regions of SM-MHC genes, including the human SM-MHC (hSM-MHC) and rat SM-MHC (rSM-MHC) genes. Prior to the instant invention, no genetic elements that are completely specific for

15 SMC and which have been proven to confer smooth muscle specific gene expression *in vivo* have been defined, isolated or identified. For example, the previously characterized SMC gene promoters, e.g., SM 22 $\alpha$  and SM  $\alpha$ -actin promoters, all show activity in both SMC and non-SMC.

The invention also provides methods for using the SM-MHC promoters and other regulatory elements to control the expression of protein and RNA products in SMC. SM-MHC

20 promoters and other regulatory elements have a variety of uses including, but not limited to, expressing heterologous genes in SMC tissues, such as the contractile portion of the stomach, intestine and uterus, the walls of arteries, the ducts of secretory glands and many other regions in which slow and sustained contractions are needed. In addition, the targeted delivery is useful for development of animal models of human disease to assist in development of new therapeutic

25 targets or development of animal models for purpose of screening new drugs/therapies.

Another aspect of the invention relates to the use of SM-MHC promoters and other regulatory elements for genetic engineering as a means to investigate SMC physiology and pathophysiology. For example, a specific gene that is believed to be important for a specific disease within SMC could be knocked out without the confounding influences of knocking out that

30 gene in other cell types and tissues. For example, an antisense polynucleotide could be expressed under the control of an SM-MHC promoter that would inhibit a target gene of interest, or an inhibitor could be expressed that would specifically inhibit a particular protein.

The conventional (non-targeted) methods for gene knockout results in embryonic lethality, thus precluding the utility of studying involvement of these genes in control of SMC differentiation in diseases such as atherosclerosis, hypertension, and asthma. With the methods of the present invention, one could examine how selective (SMC- specific) knockout of an SMC gene 5 of interest affects development of coronary artery disease without the confounding limitations of conventional knockouts with respect to deducing the primary site of action, activation of compensatory pathways, etc. Utilizing the SMC specific expression vectors of the present invention, SMC specific gene knockout can be carried out using methods known in the art. The feasibility of these sorts of approaches has been shown in other non-SMC tissue types (see, e.g., 10 Mayford *et al.*, *Science* 274:1678, 1996). For example, the SM-MHC promoter/enancers of the instant invention can be used in combination with the tetracycline-cre-recombinase based mouse 15 systems to effectuate targeted knockouts of various genes which are implicated in the control of SMC differentiation within SMC tissues (Hautmann *et al.*, *Circ. Res.* 81:600,1997; Blank *et al.*, *Circ. Res.* 76:742, 1995; Madsen *et al.*, *J. Biol. Chem.* 272:6332,1997). Examples of such genes 20 include genes which encode for serum response factor (SRF) (Kumar *et al.*, *J Biochem*, 118: 1285- 92, 1995), the homeodomain protein MHox and the retinoic acid  $\alpha$ -receptor.

A major biomedical application of the methods for SMC targeted gene delivery is to use the SM-MHC regulatory region to over-express a gene of interest within SMC. For example, an inhibitor of a pathologic process within an SMC tissue may be over-expressed in order to 20 generate a high, local concentration of the factor that might be needed for a therapeutic effect. Since expression of the gene would be SMC-specific, undesired side effects on other tissues that often result when conventional systemic administration of therapeutic agents are utilized would be avoided. For example, a gene for an SMC relaxant could be over-expressed within bronchiolar SMC as a therapy for asthma, or an inhibitor of SMC growth could be over-expressed to prevent 25 development of atherosclerosis or post-angioplasty restenosis. Such applications of the present invention is exemplified in various embodiments disclosed herein. For example, Figure 7 shows that a transgene under the control of an SM-MHC promoter was specifically expressed at high levels within all coronary arteries and arterioles within the heart of an adult mouse, demonstrating efficacy of the SM-MHC promoter/enancer for gene therapy for coronary artery disease.

30 The present invention also provides SM-MHC promoter/enancers that retain high level SMC specific expression *in vivo* and are selectively active in subsets of SMC. Expression vectors containing such promoters have tremendous utility for targeting gene expression to specific subtypes of smooth muscle *in vivo*. For example, these vectors can be employed in targeting

expression of a therapeutic gene to the specific subtype of SMC desired (e.g. bronchiolar SMC for treatment of asthma or chronic bronchitis) thereby increasing the efficacy of the therapy and reducing potential side effects due to over-expression in undesired tissues and cells. Efficacy of such applications of the present invention is demonstrated in, e.g., Figs. 4-6 and 8, which showed  
5 that some SM-MHC promoters exhibit very high activity in subsets of SMC without loss of cell specificity.

Moreover, the SM specific promoter/enancers sequences and expression vectors of the present invention can also be employed in identification and/or selection of smooth muscle cells derived from multi-potential stem cell populations for purposes of tissue generation/regeneration  
10 for surgery (e.g. for blood vessel, bladder, or gastrointestinal smooth muscle tissue augmentation-reconstitution), and/or as a means of delivering a therapeutic gene to SMC tissues *in vivo* (as described in U.S. Provisional Application No. 60/277,202). The latter involves (i) introduction of a therapeutic gene into stem cells derived from a subject's bone marrow, adipose tissue or cryo-preserved umbilical vessels; (ii) isolation and purification of SMC populations from multi-potential  
15 stem cells using the SM-MHC promoter derivatives described herein to drive expression of drug selectable markers such as puromycin; and (iii) surgical introduction of the stem-cell-derived SMC into the desired site of action *in vivo*.

A number of advantages are provided by the targeting methods of present invention. For example, SMC targeting will permit attainment of higher local concentrations of a therapeutic  
20 gene/agent at the desired site of action (i.e., SMC) than possible with systemic delivery methods, thus resulting in a greater therapeutic benefit and fewer possible side effects. In addition, SMC targeted gene therapy systems are much safer than simple "restricted access" gene delivery based methods that employ constitutively-active viral promoters, because the latter involve potential accidental delivery of a therapeutic gene to an unintended tissue or cell type may result in major  
25 undesirable side effects and possible death. By contrast, an SMC specific promoter based targeting system is superior in that even if the therapeutic gene is delivered to an undesired cell type, it will not be expressed.

The following sections provide guidance for making and using the compositions of the invention, and for carrying out the methods of the invention.

30

## II. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention

pertains. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY (2d ed. 1994); THE CAMBRIDGE DICTIONARY OF SCIENCE AND TECHNOLOGY (Walker ed., 1988); and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY (1991). Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. The following definitions are provided to assist the reader in the practice of the invention.

5 The terms "allele" or "allelic sequence" refer to an alternative form of a polynucleotide sequence. Alleles result from mutations (*i.e.*, changes in the polynucleotide sequence), and can produce differently regulated mRNAs. Common mutational changes that give rise to alleles are generally ascribed to natural deletions, additions, or substitutions of nucleotides. 10 Each of these types of changes may occur alone, in combination with the others, or one or more times within a given gene, chromosome or other cellular polynucleotide.

15 The term "amplifying" incorporates its common usage and refers to the use of any suitable amplification methodology for generating or detecting recombinant or naturally expressed polynucleotide, as described in detail, below. For example, the invention provides methods and reagents (*e.g.*, specific oligonucleotide PCR primer pairs) for amplifying (*e.g.*, by PCR) naturally expressed or recombinant polynucleotides of the invention (*e.g.*, SM-MHC promoter/enhancer sequences) *in vivo* or *in vitro*. An indication that two polynucleotides are "substantially identical" 20 can be obtained by amplifying one of the polynucleotides with a pair of oligonucleotide primers or pool of degenerate primers (*e.g.*, fragments of an SM-MHC promoter/enhancer sequence) and then using the product as a probe under stringent hybridization conditions to isolate the second sequence (*e.g.*, the SM-MHC promoter/enhancer sequence) from a genomic library or to identify the second sequence in, *e.g.*, a Northern or Southern blot.

25 A polynucleotide is "expressed" when a DNA copy of the polynucleotide is transcribed into RNA.

An "expression vector" is a polynucleotide construct, generated recombinantly or synthetically, with a series of specified polynucleotide elements that permit transcription of a particular polynucleotide in a host cell. The expression vector can be part of a plasmid, virus, or 30 polynucleotide fragment. Typically, the expression vector includes a polynucleotide to be transcribed operably linked to a promoter.

The term "heterologous" when used with reference to portions of a polynucleotide, indicates that the polynucleotide comprises two or more subsequences which are not found in the

same relationship to each other in nature. For instance, the polynucleotide is typically recombinantly produced, having two or more sequences from unrelated genes arranged in a manner not found in nature; *e.g.*, an SM-MHC promoter sequence of the invention operably linked to a polypeptide coding sequence that are not transcribed from the SM-MHC genomic locus. For 5 example, the invention provides recombinant constructs (expression cassettes, vectors, viruses, and the like) comprising various combinations of promoters of the invention, or subsequences thereof, and heterologous coding sequences, many examples of which are described in detail below.

The terms "identical" or percent "identity," in the context of two or more 10 polynucleotides or polypeptide sequences, refer to two or more sequences or subsequences that are 15 the same or have a specified percentage of nucleotides (or amino acid residues) that are the same, when compared and aligned for maximum correspondence over a comparison window, as measured 20 using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition also refers to the complement of a sequence. For example, in alternative embodiments, polynucleotides within the scope of the invention include those with a nucleotide sequence identity that is at least about 60%, at least about 75-80%, about 90%, and about 95% of the exemplary SM-MHC promoter/enhancer sequence set forth in SEQ ID NO:16 or SEQ ID NO:17, and the intronic SM-MHC sequences capable of driving a reporter gene in SM cells, as described below. Two sequences with these levels of identity are "substantially identical." Thus, if a sequence has the requisite sequence identity to an SM-MHC promoter/enhancer sequence 25 or subsequence of the invention, it also is an SM-MHC promoter/enhancer sequence within the scope of the invention.

For sequence comparison, typically one sequence acts as a reference sequence, to 30 which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated or default program parameters. A "comparison window" includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 25 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for

comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, 5 FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, *e.g.*, Ausubel et al., *supra*).

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show

10 relationship and percent sequence identity. It also plots a tree or dendrogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35:351-360 (1987). The method used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most 15 similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating 20 specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. Using PILEUP, a reference sequence (*e.g.*, an SM-MHC promoter/enhancer sequence of the invention as set forth by *e.g.*, SEQ ID NO:16 or SEQ ID NO:17) is compared to another sequence to determine the percent sequence identity relationship (*i.e.*, that the second sequence is substantially identical and within the scope of 25 the invention) using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG sequence analysis software package, *e.g.*, version 7.0 (Devereaux (1984) *Nuc. Acids Res.* 12:387-395).

Another example of algorithm that is suitable for determining percent sequence identity (*i.e.*, substantial similarity or identity) is the BLAST algorithm, which is described in 30 Altschul (1990) *J. Mol. Biol.* 215:403-410. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or

satisfy some positive-valued threshold score  $T$  when aligned with a word of the same length in a database sequence.  $T$  is referred to as the neighborhood word score threshold (Altschul (1990) *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters  $M$  (reward score for a pair of matching residues; always  $> 0$ ) and  $N$  (penalty score for mismatching residues, always  $< 0$ ). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity  $X$  from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters  $W$ ,  $T$ , and  $X$  determine the sensitivity and speed of the alignment. In one embodiment, to determine if a polynucleotide sequence is within the scope of the invention, the BLASTN program (for nucleotide sequences) is used incorporating as defaults a wordlength ( $W$ ) of 11, an expectation ( $E$ ) of 10,  $M=5$ ,  $N=4$ , and a comparison of both strands. For amino acid sequences, the BLASTP program uses as default parameters a wordlength ( $W$ ) of 3, an expectation ( $E$ ) of 10, and the BLOSUM62 scoring matrix (see, *e.g.*, Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877). One measure of similarity provided by the BLAST algorithm is the smallest sum probability ( $P(N)$ ), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a polynucleotide is considered similar to a reference sequence if the smallest sum probability in a comparison of the test polynucleotide to the reference polynucleotide is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

The term "isolated," when referring to a molecule or composition, such as, *e.g.*, an SM-MHC promoter/enhancer sequence, means that the molecule or composition is separated from at least one other compound, such as a protein, DNA, RNA, or other contaminants with which it is associated *in vivo* or in its naturally occurring state. Thus, a polynucleotide sequence is considered isolated when it has been isolated from any other component with which it is naturally associated. An isolated composition can, however, also be substantially pure. An isolated composition can be in a homogeneous state. It can be in a dry or an aqueous solution. Purity and homogeneity can be

determined, e.g., using analytical chemistry techniques such as, e.g., polyacrylamide gel electrophoresis (PAGE), agarose gel electrophoresis or high pressure liquid chromatography (HPLC).

The term "modulate" refers to the suppression, enhancement or induction of a function. For example, an agent or compound may modulate an SM-MHC promoter/enhancer sequence by binding to a motif within the promoter/enhancer, thereby enhancing or suppressing transcription of a gene operably linked to the promoter/enhancer. Alternatively, modulation may include inhibition of transcription of a gene where the an agent or compound binds to the structural gene and blocks DNA dependent RNA polymerase from reading through the gene, thus inhibiting transcription of the gene. The structural gene may be a normal cellular gene or an oncogene, for example. Alternatively, modulation may include inhibition of translation of a mRNA transcript.

The terms "nucleic acid" and "polynucleotide" are used interchangeably, and include oligonucleotides (*i.e.*, short polynucleotides). They also refer to synthetic and/or non-naturally occurring polynucleotides (*i.e.*, comprising polynucleotide analogues or modified backbone residues or linkages). The terms also refer to deoxyribonucleotide or ribonucleotide oligonucleotides in either single- or double-stranded form. The terms encompass polynucleotides containing known analogues of natural nucleotides. The term also encompasses polynucleotide-like structures with synthetic backbones. DNA backbone analogues provided by the invention include phosphodiester, phosphorothioate, phosphorodithioate, methyl-phosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene (methylimino), 3'-N-carbamate, morpholino carbamate, and peptide polynucleotides (PNAs); see Oligonucleotides and Analogues, a Practical Approach, edited by F. Eckstein, IRL Press at Oxford University Press (1991); Antisense Strategies, Annals of the New York Academy of Sciences, Volume 600, Eds. Baserga and Denhardt (NYAS 1992); Milligan (1993) J. Med. Chem. 36:1923-1937; Antisense Research and Applications (1993, CRC Press). PNAs contain non-ionic backbones, such as N-(2-aminoethyl) glycine units. Phosphorothioate linkages are described in WO 97/03211; WO 96/39154; Mata (1997) Toxicol. Appl. Pharmacol. 144:189-197. Other synthetic backbones encompassed by the term include methyl-phosphonate linkages or alternating methylphosphonate and phosphodiester linkages (Strauss-Soukup (1997) Biochemistry 36:8692-8698), and benzyl-phosphonate linkages (Samstag (1996) Antisense Nucleic Acid Drug Dev 6:153-156).

The term "operably linked" refers to a functional relationship between two or more polynucleotide (*e.g.*, DNA) segments. Typically, it refers to the functional relationship of a transcriptional regulatory sequence to a transcribed sequence. For example, an SM-MHC

- promoter/enhancer sequence of the invention, including any combination of *cis*-acting transcriptional control elements, is operably linked to a coding sequence if it stimulates or modulates the transcription of the coding sequence in an appropriate host cell or other expression system. Generally, promoter transcriptional regulatory sequences that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, *i.e.*, they are *cis*-acting. However, some transcriptional regulatory sequences, such as enhancers, need not be physically contiguous or located in close proximity to the coding sequences whose transcription they enhance. A polylinker provides a convenient location for inserting coding sequences so the genes are operably linked to the SM-MHC promoter. Polylinkers are polynucleotide sequences that comprise a series of three or more closely spaced restriction endonuclease recognition sequences.

The promoter region of a gene includes the regulatory elements that typically lie 5' to a structural gene. If a gene is to be activated, proteins known as transcription factors attach to the promoter region of the gene. This assembly resembles an "on switch" by enabling an enzyme to transcribe a second genetic segment from DNA into RNA. In most cases the resulting RNA molecule serves as a template for synthesis of a specific protein; sometimes RNA itself is the final product. The promoter region may be a normal cellular promoter or an oncopromoter.

The term "recombinant" refers to a polynucleotide synthesized or otherwise manipulated *in vitro* (*e.g.*, "recombinant polynucleotide"), to methods of using recombinant polynucleotides to produce gene products in cells or other biological systems, or to a polypeptide ("recombinant protein") encoded by a recombinant polynucleotide. "Recombinant means" also encompass the ligation of polynucleotides having coding or promoter sequences from different sources into an expression cassette or vector for expression of, *e.g.*, a fusion protein; or, inducible, constitutive expression of a protein (*i.e.*, an SM-MHC promoter/enhancer of the invention operably linked to a heterologous nucleotide, such as a polypeptide coding sequence).

The "sequence" of a gene (unless specifically stated otherwise) or polynucleotide refers to the order of nucleotides in the polynucleotide, including either or both strands of a double-stranded DNA molecule, *e.g.*, the sequence of both the coding strand and its complement, or of a single-stranded polynucleotide molecule. For example, the promoter of the invention comprises untranscribed, untranslated, and intronic SM-MHC sequences, *e.g.*, as set forth in the exemplary SEQ ID NO:16 and SEQ ID NO:17.

Unless otherwise specified, the term "SM-MHC" broadly refers to smooth muscle myosin heavy chain, as well as the corresponding polynucleotide and polypeptide sequences. See White et al., J. Biol. Chem. 27115008-15017, 1996.

Unless otherwise specified, the terms "SM-MHC promoter," "SM-MHC promoter/enhancer" and "SM-MHC promoter/enhancer sequence" are used interchangeably and refer to a polynucleotide which comprises SM-MHC genomic sequence and activates transcription of a linked polynucleotide in smooth muscle cells *in vitro* and *in vivo*. Unless otherwise noted, the 5 SM-MHC promoter/enhancers of the present invention do not include a polynucleotide which can drive DNA expression in cultured SMCs, but not in an animal having a smooth muscles (e.g., transgenic mice). The SM-MHC promoter/enhancer sequences can include all *cis*-acting SM-MHC transcriptional control elements and regulatory sequences, including (without limitation) those that regulate and modulate timing and rates of transcription. Thus, the SM-MHC promoter/enhancer 10 sequences of the invention can include *cis*-acting elements such as, *e.g.*, promoters, enhancers, transcription terminators, origins of replication, chromosomal integration sequences, introns, exons, and 5' and 3' untranslated regions, with which proteins or other biomolecules interact to carry out and regulate transcription of the SM-MHC transcript.

15 The term "smooth muscle-specific expression" or "smooth muscle-specific transcription" means that a polynucleotide is transcribed at a greater rate in smooth muscle cells than in non-smooth muscle cells. Exemplary SM cells include cells which form the contractile portion of the stomach, intestine and uterus, the walls of arteries, the ducts of secretory glands and many other regions in which slow and sustained contractions are needed. In general, an SM specific promoter and/or enhancer will generally activate transcription of a linked polynucleotide at 20 least 3-fold more efficiently in SM cells than in non-SM cells. In certain embodiments, transcription is at least 3-fold, 5-fold, 10-fold, 25-fold or 100-fold more efficient in SM cells than in non-SM cells. Unless otherwise specified, SM-MHC promoter/enhancers of the present invention do not have detectable activity in non-SM cells when examined using a reporter gene (*e.g.*, lacZ) as described in the *Examples*. SM-specific transcription may result from an increased frequency of 25 transcriptional initiation, an increased rate of transcriptional elongation, a decreased frequency of transcriptional termination, or a combination thereof.

The phrase "selectively (or specifically) hybridizes to" refers to the binding, duplexing, or hybridizing of a molecule to a particular nucleotide sequence under moderately or highly stringent hybridization conditions when that sequence is present in a complex mixture (*e.g.*, 30 total cellular or library DNA or RNA), wherein the particular nucleotide sequence is detected at least twice background, preferably 10 times background. In one embodiment, a polynucleotide can be determined to be within the scope of the invention (*e.g.*, is substantially identical to an SM-MHC promoter/enhancer of the invention, as exemplified by SEQ ID NO:16 or SEQ ID NO:17, or, by an

intronic promoter sequence, as described below) by its ability to hybridize under stringent conditions to another polynucleotide (such as the exemplary sequences described herein).

The phrase "stringent hybridization conditions" refers to conditions under which a probe will primarily hybridize to its target subsequence, typically in a complex mixture of 5 polynucleotide, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances, *e.g.*, depending on the length of the probe. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of polynucleotides is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--* Hybridization with Nucleic Probes, "Overview of principles of hybridization and the strategy of 10 nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 15 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at Tm, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium 20 ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (*e.g.*, 10 to about 50 nucleotides) and at least about 60°C for long probes (*e.g.*, greater than about 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific 25 hybridization, a positive signal (*e.g.*, identification of a polynucleotide of the invention) is about 5-10 times background hybridization. "Stringent" hybridization conditions that are used to identify substantially identical polynucleotides within the scope of the invention include hybridization in a buffer comprising 50% formamide, 5x SSC, and 1% SDS at 42°C, or hybridization in a buffer comprising 5x SSC and 1% SDS at 65°C, both with a wash of 0.2x SSC and 0.1% SDS at 65°C, for 30 long probes. For short probes, stringent hybridization conditions include hybridization in a buffer comprising 50% formamide, 5xSSC and 1% SDS at room temperature or hybridization in a buffer comprising 5 xSSC and 1% SDS at 37° C- 42°C, both with a wash of 0.2 x SSC and 0.1% SDS at 37° C- 42°C. However, as is apparent to one of ordinary skill in the art, hybridization conditions can be modified depending on sequence composition. Exemplary "moderately stringent" hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, and 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

“Transcription initiation elements” refer to sequences in a promoter that specify the start site of RNA polymerase II. Transcription initiation elements may include TATA boxes, which direct initiation of transcription 25-35 bases downstream, or initiator elements, which are sequences located near the transcription start site itself. Eukaryotic promoters generally comprise

5 transcription initiation elements and either promoter-proximal elements, distant enhancer elements, or both. SM-MHC transcription initiation elements may include the TATA box or transcription initiation sites described herein, or both. Heterologous transcription initiation elements may be obtained from any eukaryotic promoter, although mammalian and viral promoters are preferred sources of heterologous initiation elements.

10 The term “transcribable sequence” refers to any sequence which, when operably linked to a *cis*-acting transcriptional control element, *e.g.*, a promoter, such as the SM-MHC promoter/enhancers of the invention, and when placed in the appropriate conditions, is capable of being transcribed to generate RNA, *e.g.*, messenger RNA (mRNA).

### 15 III. Polynucleotides Comprising Smooth Muscle Specific Promoter/Enhancers

The present invention provides polynucleotide sequences which confer to an operably linked polynucleotide cell-specific expression within SM cells *in vivo*. These polynucleotide sequences, termed SM-MHC promoter/enhancers, are derived from the smooth muscle myosin heavy chain (SM-MHC) gene. Some of the SM-MHC promoter/enhancers are obtained from the human SM-MHC sequence (*e.g.*, SEQ ID NO:17). SEQ ID NO:17 contains residues -5086 to +13,518 of the human SM-MHC gene sequence. Nucleotide 1 in SEQ ID NO:17 corresponds to position -5086 relative to the transcription start site (+1 position) which in turn corresponds to position 143,590 in the undefined BAC sequence contained in the public database (GenBank Accession No. U91323). Some of the SM-MHC promoter/enhancers are derived from 20 rat SM-MHC sequence (*e.g.*, SEQ ID NO:16). Nucleotide 1 of SEQ ID NO:16 corresponds to position -4,216 bp relative to the SM-MHC transcription start site.

The present invention also provides SM specific promoter/enhancers which are active only in certain subsets of smooth muscle tissues. Some of these SM-MHC promoter/enhancers comprise a polynucleotide sequence which consists essentially of the region of 25 nucleotides 5663 to 5889 of SEQ ID NO:16 (the +1447 to +1673 intronic region). Other comprise a sequence of SEQ ID NO:16 except that the CArG2 or the intronic CArG motif has been mutated. Some of the promoter/enhancers comprise a polynucleotide sequence which consists essentially of the regions of nucleotides 1 to 6,700 (the -4.2 to +2.5 region) and nucleotides 9,500 to 15,800 (the

+5.3 to +11.6 region) of SEQ ID NO:16. Some of the subset-specific SM-MHC promoter/enhancers comprise a polynucleotide sequence which consists essentially of the regions of nucleotides 1 to 9,500 (the -4.2 to +5.3 region) and nucleotides 11,700 to 13,700 (the +7.5 to +9.5 region) of SEQ ID NO:16.

5 In alternative embodiments, the SM-MHC promoter/enhancer sequences comprise sequences substantially identical to an exemplary SM-MHC promoter/enhancer sequence as discussed above. Thus, SM-MHC promoters/enhancers of the instant invention include homologous SMC promoter/enhancer elements which have similar functional activity. This includes SMC promoters/enhancers which direct SMC-specific expression in vivo and either 10 hybridize to the above-described SM-MHC promoter/enhancers under highly stringent conditions, or that hybridize to the complement of the above-described promoter/enhancers under moderately stringent conditions.

15 SM-MHC promoter/enhancer sequences can range from 100 to 20,000 nucleotides in length, although in particular embodiments functional SM-MHC promoter/enhancer polynucleotides may be at least or no more than about 300, 500, 1,000, 2,500, 5,000, 10,000, or 15,000 nucleotides in length. SM-MHC promoter/enhancer polynucleotides of the present invention are generally at least 70% homologous to SEQ ID NO:16 or SEQ ID NO:17 over a stretch of 150 nucleotides or more. In some embodiments, SM-MHC promoter/enhancer polynucleotides are at least 75%, 80%, 85%, 90%, 92%, 95%, or 100% homologous to SEQ ID 20 NO:16 or SEQ ID NO:17 over a stretch of 300, 500, 1,000, 2,500, 5,000, 10,000 or 15,000 nucleotides.

25 As detailed in the Examples, some of the SM-MHC promoter/enhancer sequences comprise non-transcribed SM-MHC genomic sequence as well as either SM-MHC introns or exons, or both. In some embodiments, SM-MHC promoter/enhancer polynucleotides include the SM-MHC TATA box and transcription initiation sites (collectively referred to as SM-MHC transcription initiation elements). In embodiments where the SM-MHC transcription initiation elements are the only functional initiation elements of the promoter, the natural orientation of the SM-MHC TATA box or transcription initiation sites, relative to the direction of transcription, should be preserved. In other embodiments, SM-MHC promoter/enhancer polynucleotides are 30 connected to heterologous TATA boxes and/or transcription initiation sites. When linked to heterologous TATA boxes or transcription initiation sites, SM-MHC promoter/enhancer polynucleotides act as enhancer elements and may be inserted in either orientation relative to the direction of transcription. Thus, the term "SM-MHC promoter/enhancer" encompasses

polynucleotides comprising the transcription initiation elements of the SM-MHC gene, as well as cis-linked enhancer sequences that yield smooth muscle-specific expression when linked to the transcription initiation elements of a heterologous gene.

5           A.    Isolation of SM specific promoter/enhancer sequences

1           1.    Isolation of SM-MHC promoter sequences

The SM-MHC promoter/enhancer sequences of the invention and polynucleotides used to practice this invention, whether RNA, cDNA, genomic DNA, or hybrids thereof, may be isolated from a variety of sources, genetically engineered, amplified, and/or expressed recombinantly. Any recombinant expression system can be used, including, e.g., bacterial, yeast, insect or mammalian systems. Alternatively, these polynucleotides can be chemically synthesized *in vitro*.

In some embodiments, SM-MHC promoter sequences are isolated from libraries of genomic DNA. Some genomic libraries are commercially available. For example, rat genomic phage library can be obtained from Stratagene Corp. Genomic DNA libraries are also available from various other commercial suppliers (e.g., Incyte Genomics, Palo Alto, CA; Clontech, Palo Alto, CA). Alternatively, genomic libraries can also be constructed, e.g., as described in Ausubel et al., *supra*. For a genomic library, the DNA is extracted from the tissue and either mechanically sheared or enzymatically digested to yield fragments of about 12-20 kb. The fragments are then separated by gradient centrifugation from undesired sizes and are constructed in bacteriophage lambda vectors. These vectors and phage are packaged *in vitro*. Recombinant phage are analyzed by plaque hybridization as described in Benton & Davis, *Science* 196:180-182 (1977). Colony hybridization is carried out as generally described in Grunstein et al., *Proc. Natl. Acad. Sci. U.S.A.*, 72:3961-3965 (1975).

25           In some embodiments, the SM-MHC promoter/enhancer sequences are obtained from genomic clones containing 5' flanking region and the intronic regions of the SM-MHC gene. Standard methods that may be used in such screening include, for example, the method set forth in Benton & Davis, 1977, *Science* 196:180 for bacteriophage libraries; and Grunstein & Hogness, 1975, *Proc. Nat. Acad. Sci. U.S.A.* 72:3961-3965 for plasmid libraries.

30           SM-MHC promoter polymorphic variants, orthologs, and alleles that are substantially identical to SM-MHC promoter sequences can be isolated using SM-MHC promoter/enhancer polynucleotide probes and oligonucleotides under stringent hybridization conditions, by screening libraries from the appropriate organism.

Techniques for the manipulation of polynucleotides, such as, *e.g.*, subcloning into expression vectors, labeling probes, sequencing, and hybridization are well described in the scientific and patent literature, see *e.g.*, ed., Molecular Cloning: A Laboratory Manual (2nd Ed.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) ("Sambrook"); Current Protocols In Molecular 5 Biology, Ausubel, ed. John Wiley & Sons, Inc., New York (1997) ("Ausubel"); Laboratory Techniques In Biochemistry And Molecular Biology: Hybridization With Nucleic Acid Probes, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993) ("Tijssen"). Nucleic acids can be analyzed and quantified by any of a number of general means well known to those of skill in the art. These include, *e.g.*, analytical biochemical methods such as NMR, 10 spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high pressure liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography, various immunological methods, such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immuno-fluorescent assays, Southern analysis, Northern analysis, dot-blot 15 analysis, gel electrophoresis (*e.g.*, SDS-PAGE), RT-PCR, quantitative PCR, other polynucleotide or target or signal amplification methods, radiolabeling, scintillation counting, and affinity chromatography.

20 Oligonucleotides that are not commercially or publicly available can be chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, *Tetrahedron Letts.* 22:1859-1862 (1981), using an automated synthesizer, as described in Van Devanter *et. al.*, *Nucleic Acids Res.* 12:6159-6168 (1984). Purification of 25 oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.* 255:137-149 (1983).

Synthetic oligonucleotides can be also used to construct recombinant SM-MHC 30 promoter sequences for use as probes or for generation of smooth muscle-specific promoters. This method is performed using a series of overlapping oligonucleotides usually 40-120 bp in length, representing both the sense and non-sense (antisense) strands of the gene. These DNA fragments are then annealed, ligated and cloned. Alternatively, amplification techniques can be used with precise primers to amplify a specific subsequence of an SM-MHC promoter sequence.

SM-MHC promoter sequences are typically cloned into intermediate vectors before transformation into prokaryotic or eukaryotic cells for replication and/or expression. These intermediate vectors are typically prokaryotic vectors, *e.g.*, plasmids, or shuttle vectors.

2. Modification of SM-MHC promoter sequences

Once smooth muscle-specific transcriptional activity has been demonstrated in an SM-MHC promoter/enhancer sequence, deletions, mutations, rearrangements, and other sequence modifications may be constructed and analyzed for smooth muscle-specific transcription. Such 5 derivatives of SM-MHC promoter sequences are useful to generate more compact promoters, to decrease background expression in non-smooth muscle cells, to eliminate repressive sequences, or to identify novel smooth muscle-specific transcriptional regulatory proteins.

SM-MHC promoter subfragments and derivatives may be constructed by conventional recombinant DNA methods known in the art. One such method is to generate a series 10 of deletion derivatives within the promoter sequence (see, e.g., Fig. 18A and Example 2). By comparing the transcriptional activity of a deletion series, the elements that contribute to or detract from smooth muscle-specific transcription may be localized. Based on such analyses, improved derivatives of SM-MHC promoter sequences may be designed. SM-MHC promoter elements may be combined with smooth muscle-specific or ubiquitous regulatory elements from heterologous promoters to increase the specificity or activity of an SM-MHC promoter sequence.

The modified SM-MHC promoter/enhancer sequences can contain deletion in one or more of the *cis*-acting elements. *Cis*-acting regulatory elements within a promoter/enhancer may be identified using methods such as DNase or chemical footprinting (e.g. Meier *et al.*, 1991, *Plant Cell* 3:309-315) or gel retardation (e.g., Weissenbom & Larson, 1992, *J. Biol. Chem.* 267:6122-6131; Beato, 1989, *Cell* 56:335-344; Johnson *et al.*, 1989, *Ann. Rev. Biochem.* 58:799-839). 20 Additionally, resectioning experiments also may be employed to define the location of the *cis*-regulatory elements. For example, a promoter/enhancer containing fragment may be resected from either the 5' or 3' end using restriction enzyme or exonuclease digests.

In addition, specific base pairs can be modified to alter, increase or decrease the 25 binding affinity to trans-acting transcriptional regulatory factors, thus modifying the relative level of transcriptional activation or repression. Modifications can also change secondary structures of specific subsequences, such as those associated with many *cis*-acting transcriptional elements. Site-specific mutations can be introduced into polynucleotides by a variety of conventional techniques, well described in the scientific and patent literature. Illustrative examples include, e.g., 30 site-directed mutagenesis by overlap extension polymerase chain reaction (OE-PCR), as described in Urban (1997) *Nucleic Acids Res.* 25:2227-2228; Ke (1997) *Nucleic Acids Res* 25:3371-3372, and Chattopadhyay (1997) *Biotechniques* 22:1054-1056. Modified SM-MHC promoter/enhancer sequences of the invention can be further produced by chemical modification methods, see, e.g.,

Belousov (1997) Nucleic Acids Res. 25:3440-3444; Frenkel (1995) Free Radic. Biol. Med. 19:373-380; Blommers (1994) Biochemistry 33:7886-7896.

5           B.     Activity of SM-MHC promoter/enhancers

The present invention provides smooth muscle-specific SM-MHC promoters and enhancers. Accordingly, methods for assaying the smooth muscle-specific transcription induced by SM-MHC promoter sequences are provided herein.

10           Promoter activity of an SM-MHC promoter sequence is generally assayed by operably linking the SM-MHC promoter sequence to a reporter gene (e.g., a *lacZ* gene) in a test construct (see, e.g., Example 1, *infra*). When inserted into the appropriate host cell (e.g., cultured rat SM cells), the SM-MHC promoter sequence induces transcription of the reporter gene by host RNA polymerases. Reporter genes typically encode proteins (e.g.,  $\beta$ -galactosidase) with an easily assayed enzymatic activity that is naturally absent from the host cell. Alternatively, endogenous activity of the reporter protein can be measured with a control construct which does not express the reporter gene, and subtracted from the activity measured for the test construct. In addition to  $\beta$ -galactosidase, other reporter proteins that can be applied in the present invention include chloramphenicol acetyltransferase (CAT), firefly or *Renilla* luciferase,  $\beta$ -galactosidase, beta- $\beta$ glucuronidase, alkaline phosphatase, and green fluorescent protein (GFP). In some embodiments, SM-MHC promoter fragments can be inserted into a polylinker sequence and tested for activity of the reporter protein in the appropriate host cell (see, e.g., U.S. Patent No. 5,670,356).

20           Transcription driven by SM-MHC promoter sequences can also be detected by directly measuring the amount of RNA transcribed from the reporter gene. In these embodiments, the reporter gene can be any transcribable polynucleotide of known sequence that is not otherwise expressed by the host cell. RNA expressed from SM-MHC promoter constructs may be analyzed by techniques known in the art, e.g., reverse transcription and amplification of mRNA, isolation of 25 total RNA or poly A<sup>+</sup> RNA, northern blotting, dot blotting, *in situ* hybridization, RNase protection, primer extension, high density polynucleotide array technology and the like.

30           In addition to reporter genes, vectors for assaying SM-MHC promoter sequence activity also comprise elements necessary for propagation or maintenance in the host cell, and elements such as polyadenylation sequences and transcriptional terminators to increase expression of reporter genes or prevent cryptic transcriptional initiation elsewhere in the vector. Assay vectors may also comprise other transcription regulatory (e.g., transcription initiation) sequences,

depending on whether the SM-MHC transcription initiation elements are included in the SM-MHC promoter sequence being assayed.

1. Assaying activity of SM-MHC promoter/enhancers SM cells

5 The ability of a promoter sequence to activate transcription can be assessed relative to a control construct which harbors a reference promoter. In some embodiments, the specificity of an SM-MHC promoter sequence to activate transcription is assessed by comparing the expression of a reporter gene operably linked to an SM-MHC promoter sequence with the expression of the identical reporter gene operably linked to a reference promoter. For example, the activity of a  
10 reporter gene driven by an SM-MHC promoter sequence can be compared to the activity of a reporter gene driven by a characterized promoter (e.g., the SV40 promoter/enhancer, Promega, Madison, WI).

20 SM-MHC promoter sequences of the present invention are smooth muscle-specific, activating transcription to a greater extent in smooth muscle cells than in non-smooth muscle cells. Accordingly, smooth muscle specificity of an SM-MHC promoter sequence may be assessed by assaying its promoter or enhancer activity in a smooth muscle cell and a non-smooth muscle cell. In some embodiments, the assay for smooth muscle-specific promoter activity generally requires simultaneous comparison of reporter gene expression in four contexts: the test promoter in a smooth muscle cell, a reference promoter (e.g., lacking SM-MHC sequences) in the smooth muscle cell, the test promoter in a non-smooth muscle cell, and the reference promoter in a non-smooth muscle cell. Once the promoter activity of the SM-MHC polynucleotide in each cell type is determined by comparing the test promoter and the reference promoter, the smooth muscle specificity of the SM-MHC polynucleotide is calculated by comparing the activity of the test promoter in the smooth muscle cell with its activity in a non-smooth muscle cell.

25 One system for assessing SM-MHC promoter activity is transient or stable transfection into cultured cell lines. Assay vectors bearing SM-MHC promoter sequences operably linked to reporter genes can be transfected into any mammalian cell line for assays of promoter activity. Suitable methods of cell culture, transfection, and reporter gene assay are described in, e.g., Ausubel et al., *supra*; or *Transfection Guide*, Promega Corporation, Madison, WI (1998). SM-  
30 MHC promoter sequences may be assayed for smooth muscle-specific transcription activity by transfecting the assay vectors in parallel into smooth muscle cell lines and non-smooth muscle cell lines. In some embodiments, a control vector comprising a second reporter gene driven by a known promoter (e.g., *Renilla* luciferase driven by the SV40 early promoter/enhancer; pRL-SV40,

Promega, Madison, WI) is co-transfected along with the assay vector to control for variations in transfection efficiency or reporter gene translation among the smooth muscle and non-smooth muscle cell lines.

5           2.       Assaying *in vivo* activity of the SM-MHC promoter/enhancers

As disclosed above, the activity of specificity of the SM-MHC promoter/enhancers of the present invention can be assayed in eukaryotic *in vitro* transcription systems (e.g., cultured rat SM cells). Their activity can also be examined in transgenic animals (e.g., transgenic mice). Further, it is known that some promoter or enhancers with specificity in cultured SM cells do not 10 have activity *in vivo*, e.g., in transgenic mice. Thus, to determine *in vivo* specificity, the SM-MHC promoter/enhancers are also assayed for their activity in transgenic animals.

Transgenic animals (e.g., transgenic mice) expressing SM-MHC promoter/enhancer can be generated accordingly to methods well known in the art (see, e.g., Example 1). For example, techniques routinely used to create and screen for transgenic animals have been described in, e.g., see Bijvoet (1998) *Hum. Mol. Genet.* 7:53-62; Moreadith (1997) *J. Mol. Med.* 75:208-216; Tojo (1995) *Cytotechnology* 19:161-165; Mudgett (1995) *Methods Mol. Biol.* 48:167-184; Longo (1997) *Transgenic Res.* 6:321-328; U.S. Patents Nos. 5,616,491 (Mak, et al.); 5,464,764; 5,631,153; 5,487,992; 5,627,059; 5,272,071; and, WO 91/09955, WO 93/09222, WO 96/29411, WO 95/31560, and WO 91/12650.

Transgenic animals with integrated SM-MHC promoter sequences can be used to assay for SM specific transcription. In some embodiments, an SM-MHC promoter sequence, linked either to a reporter gene or to native SM-MHC coding sequence, is injected into the embryo of a developing animal (typically a mouse) to generate a transgenic animal. Once integration of the transgene has been verified, smooth muscle and non-smooth muscle tissues of the animal are then 25 assayed for expression of the transgene with conventional RNA or protein detection methods known in the art and described herein. Typically, a rat or a human SM-MHC promoter sequence is employed, in which case RNA expressed from the transgene may be distinguished from RNA expressed from the endogenous mouse SM-MHC locus by employing appropriate polynucleotide probes that are specific for the rat or human SM-MHC sequence. Alternatively, where the SM- 30 MHC promoter sequence is linked to a reporter gene, tissues of the transgenic animal may be assayed either for reporter gene RNA or for the enzymatic activity of the reporter protein (see, e.g., Examples 1, 2 and 4).

5 C. Exemplary SM-MHC promoter/enhancers

The SM specific promoter disclosed herein can be obtained as described in the Examples, e.g., cloned from genomic DNA libraries or isolated using amplification techniques with oligonucleotide primers. An exemplary SM specific promoter is the rat -4.2 to +11.6 region promoter/enhancer of rat SM-MHC (SEQ ID NO:16) (see also, Madsen et al., Circ. Res. 82:908-917, 1998). The corresponding human SM-MHC promoter/enhancer sequence has also been identified (the -5,086 to +13,518 fragment; SEQ ID NO:17). Other than the 5'- flanking sequence (e.g., residues 1-4216 of SEQ ID NO:16) and the short first exon (e.g., the 88 bp exon in SEQ ID NO:16), these SM-MHC promoter/enhancer sequences also contain portion of the first intron of the SM-MHC gene.

10 Some of the SM-MHC promoter/enhancers confer specificity in all SM cells. For example, the -4.2 to +11.6 kb fragment of the SM-MHC promoter/enhancer (corresponding to residues 1-16011 of SEQ ID NO:16) exhibits high level activity in virtually all SMC subtypes (Figs. 1-3 and 12). Transgene expression under control of this promoter was observed in both arterial and venous smooth muscle, airway smooth muscle of the trachea and bronchi and in the smooth muscle layers of all abdominal organs, including the stomach, intestine, ureters and bladder. In addition, the transgene was expressed at high levels throughout the coronary circulation (see, Figure 7). During development, transgene expression was first detected in airway SMC at embryonic day 12.5 and in vascular and visceral SMC tissues by embryonic day 14.5.

15 Human SM-MHC promoter/enhancer with in vivo SM specificity was also identified by the present inventors. As disclosed in Examples 2-4 and Figs. 9-10), the region from -5086 to +13518 of the human SM-MHC gene (SEQ ID NO:17) was found highly active in multiple SMC tissues, but exhibited absolutely no expression in non-SMC tissues. This included expression in SMC within multiple small and large vessels including the aorta, coronary arteries, iliac, celiac, 20 mesenteries, etc. This promoter was also robustly expressed in SMC within the stomach, intestine, bladder, ureter, and airways. As illustrated in Figures 11, 17 and 18, there is complete sequence homology between the rat and human genes in the key regulatory regions identified thus far (e.g. 5' CArG 1, 2 and 3; the G/C repressor, etc., as indicated). The identity of these elements in the rabbit and mouse genes have been shown previously (e.g., Iadsen et al., 1997, *J. Biol. Chem.*, 272:6332).

25 30 Other than conferring specificity in all SM tissues, some SM-MHC promoter/enhancers confer SM specificity only in selective subtypes of SM tissues (i.e. vascular versus gastrointestinal SMC, large versus small arteries, pulmonary versus gastrointestinal SMC, etc.) (see, Examples 2-4, Figs. 4-8). Various derivative SM-MHC promoter/enhancers obtained

from the -4.2 to +11.6 kb rat SM-MHC promoter/enhancer region were found to be active in one or more, but not all subtypes of SM tissues. For example, some of such SM-MHC promoter/enhancers include those that comprise essentially the sequence of nucleotides 5663 to 5889 of SEQ ID NO:16 (corresponding to the intronic region of +1447 to +1673). This fragment 5 contains three repeats of the intronic region of SM-MHC, and when coupled to a minimal thymidine kinase (TK) promoter, confers high level expression in multiple SMC tissues including the aorta, coronary arteries, and pulmonary artery (See Fig. 7).

Some of the subtype-specific SM-MHC promoter/enhancers have an excision of the region from +5.3 to +11.6 kb. These promoters do not confer SM expression in vascular SMC but 10 retain the activity in gastrointestinal, and bladder SMC. Some of these subtype-specific SM-MHC promoter/enhancers consists essentially the regions -4.2 to +5.3 and +7.5 to +9.5 (corresponding to residues 1 to 9,500 and nucleotides 11,700 to 13,700 of SEQ ID NO:16), or -4.2 to +2.5 and +5.3-11.6 (corresponding to residues 1 to 6,700 and nucleotides 9,500 to 15,800 of SEQ ID NO:16). They exhibit very high activity in both pulmonary vascular and airway SMC (see Figures 4-6).

In still some other SM-MHC promoter/enhancers, subtype-specificity is conferred by the deletion of certain conserved motifs (e.g., the intronic CArG motif or the CArG2 motif). For example, some of the promoter/enhancers have a mutation in the conserved intronic CArG element (i.e., residues 5815-5824 of SEQ ID NO:16). An exemplary mutant has the intronic CArG sequence changed from CCTTGTATGG (SEQ ID NO:5) to AGGCCTATGG (SEQ ID NO:6). The mutation abolishes promoter activity in the aorta, coronary arteries, and the carotid artery, without affecting expression in other SMC tissues including pulmonary vascular or airway SMC (see, e.g., Fig. 8). Some other SM-MHC promoters have a mutation in the CArG2 motif (i.e., residues 3105-3114 of SEQ ID NO:16). An exemplary promoter having such a mutation has the CArG2 sequence changed from TTCCTTTATGG (SEQ ID NO:1) to GGATCCTATGG (SEQ ID NO:2).

25

#### D. Expression vectors and transgenic animals

The invention provides expression vectors for targeted gene delivery and expression in SM cells. The expression vectors comprise an SM-MHC promoter/enhancer sequence operably linked to a heterologous gene (in a preferred embodiment, a structural gene). The heterologous 30 coding sequence operably linked to an SM-MHC promoter/enhancer of the invention can be a marker or reporter gene (e.g., alkaline phosphatase, SEAP;  $\beta$ -galactosidase), a modified SM-MHC structural gene or an SM-MHC antisense sequence, a therapeutic gene. Other than the promoter and a heterologous gene, the vectors can also comprise other elements, e.g., origins of replication.

These constructs are useful for SM-MHC promoter-based assays, for example, to identify biological modulators of SM-MHC promoter/enhancer activity.

Some of the SMC specific expression vectors of the present invention comprise an SM-MHC promoter sequence described above. Some of the expression vectors contain the 5 polynucleotide sequence of SEQ ID NO:16 or 17. Some expression vector contain an SM-MHC promoter/enhancer which consists essentially of one of the following sequences

1) the intronic region from +1447 to + 1673 (residues 5663 to 5889) of SEQ ID

NO:16;

2) the region of - 4.2 to +11.6 of SEQ ID NO:16, wherein CArG2 or intronic CArG

10 have been mutated;

3) the regions of - 4.2 to +2.5 and +5.3 to +11.6 (residues 1 to 6,700 and nucleotides 9,500 to 15,800) of SEQ ID NO:16; and

4) the regions of - 4.2 to +5.3 and +7.5 to +9.5 (residues 1 to 9,500 and nucleotides 11,700 to 13,700 of SEQ ID NO:16) of SEQ ID NO:16.

As discussed in more detail below, these expression vectors are useful for targeting gene expression specifically to smooth muscle or subtypes of smooth muscle, development of animal models of human disease for drug screening, or elucidation of pathogenic mechanisms and identification of new therapeutic targets.

Employing aforementioned expression vectors, the present invention provides host 20 cells and transgenic animals which have incorporated a heterologous polynucleotide in SM cells. Such host cells or transgenic animals (e.g., transgenic mice) of the present invention can be produced as described above and in the Examples. The transgenic cells or animals of the present invention can be used in various applications, e.g., development of animal models for purpose of 25 screening new drugs/therapies. For example, if a specific gene is known to be involved in an SMC-based disease, the gene can be operably linked to an SM-MHC promoter/enhancer of the instant invention to produce an animal model of the disease.

In addition, transgenic cells or animals of the present invention can also comprise an 30 SM-MHC promoter/enhancer operably linked to a gene which expresses a protein which can inhibit (a) other proteins or (b) transcription of other genes that further the diseased state being examined within the animal model. Alternatively, the SM-MHC promoter/enhancer can be operably linked to an antisense gene, which could specifically inhibit expression of a gene which may be involved in the diseased state. Using such animal models, one of skill in the art could test conventional drug

therapies, identify key genes involved in the development of these diseases and/or develop a novel way of curing the disease.

#### **IV. Targeted Gene Delivery And Expression**

5 The present invention provides methods for targeted delivery of therapeutic agents to SMC cells in a subject (human or non-human animals). The therapeutic agents include polynucleotides that are specifically expressed in vivo under the control of the SM-MHC promoter/enancers. Virtually any gene can be specifically expressed within SMC in the subject. The expression vectors can be introduced or reintroduced into a subject (e.g., a human patient) at 10 positions which allow for the amelioration of SMC-related disease. The subtype-specific SM-MHC promoter derivatives that are selectively active in subsets of SMC (e.g. vascular versus gastrointestinal SMC, large versus small arteries, pulmonary versus gastrointestinal SMC, etc.) enable targeted gene expression in specific subtypes of smooth muscle in vivo. Thus, advantages of the targeting methods of the present invention include complete SMC specificity, the ability to target specific SMC subsets, a small size compatible with existing gene delivery methods, and high level activity.

20 For example, the expression vectors of the present invention can be employed in targeting expression of a therapeutic gene to the specific subtype of SMC desired (e.g. bronchiolar SMC for treatment of asthma or chronic bronchitis) thereby increasing the efficacy of the therapy and reducing potential side effects due to over-expression in undesired tissues and cells. In addition, the expression vectors can also be used in development of animal models of human disease to assist in development of new therapeutic targets. Further, the expression vectors and targeting methods of the present invention can also be used in identification and/or selection of 25 smooth muscle cells derived from multi-potential stem cell populations for purposes of tissue generation/regeneration for surgery (e.g. for blood vessel, bladder, or gastrointestinal smooth muscle tissue augmentation-reconstitution).

##### **A. Diseases amenable to treatment with methods of the present invention**

30 The present invention provides compositions and methods for targeted gene delivery and expression that can be used to treat a variety of diseases and conditions. A large number of major human diseases including systemic hypertension, pulmonary hypertension, atherosclerosis, asthma, coronary artery disease, gastrointestinal abnormalities, reproductive dysfunction, and chronic bronchitis are associated with abnormal function of the smooth muscle cell (SMC). A

specific example is to target over-expression of nitric oxide synthase (the enzyme responsible for production of the SMC relaxant nitric oxide or NO from L-arginine) to bronchiolar SMC using our SM-MHC promoter derivative that is active in bronchiolar SMC but inactive in many other SMC tissues. This targeting would be critical to avoid potential deleterious effects of over-expression of NO in other SMC subtypes including vascular SMC which might be associated with severe hypotension and possible death. Similar approaches could be used to target NO synthase to arteriolar SMC as a means of treating certain forms of hypertension that are resistant to current therapies.

The present invention also find application in development of animal models of disease for purposes of testing potential new drugs/therapies, and/or identifying disease mechanisms. For example, one might over-express protease enzymes in vascular SMC in large blood vessels as a model to study development of aneurysms and ways to prevent or treat them. Additional applications of the present invention include development of gene targeting therapies for promoting formation of collateral vessels following tissue ischemia. Methods of the present invention can be used in developing ways to promote formation of collateral blood vessels in the myocardium following a non-fatal heart attack. For example, the targeting methods of the present invention can be used to over-express angiogenic substances such as VEGF in the coronary microcirculation in an ischemic heart region.

The present inventors have identified a molecular mechanism that appears to be important in mediating repression of SM cell marker genes such as SM-MHC and SM22a that occur when SM cells undergo phenotypic modulation in response to vascular injury. Specifically, a G/C repressor element was identified within the promoters of both the SM-MHC and SM22 genes. This repressor element was found to mediate suppression of the activity of these promoters in phenotypically modulated cultured SMC (see, e.g., Madsen et al., *J.Biol.Chem.* (1997) 272:6332-6340; and Madsen et al., *J.Biol.Chem.* (1997) 272:29842-29851). It was shown that mutation of the SM22a G/C element prevented injury-induced down-regulation of these genes, but did not affect the tissue selectivity of this promoter. Such an repressor element can be the target (or "useful") in SMC gene targeting applications that are associated with phenotypic modulation of SMC, e.g., post-angioplasty restenosis, intimal SMC within atherosclerotic lesions, or vascular remodeling in pulmonary hypertension, etc. For example, the SM-MHC promoter/enhancer of the present invention can be used in the context of vascular injury in which activity of the wild type SM-MHC promoter is repressed.

B. Targeted delivery of therapeutic agents

Therapeutic agents to be delivered with the targeting methods of the present invention include any therapeutic polynucleotide operably linked to an SM-MHC promoter sequence in an expression vector discussed above. Therapeutic polynucleotides (including those 5 that can be identified with the screening methods described below) expressed by SM-MHC promoter sequences are either active themselves (e.g., antisense and catalytic polynucleotides) or encode a therapeutic protein.

1. Antisense and catalytic ribonucleotides

One type of therapeutic polynucleotide that can be expressed by SM-MHC promoter sequences is antisense RNA. In such embodiments, the SM-MHC promoter sequence is operably linked to a polynucleotide which, when transcribed by cellular polymerases, is capable of binding to target mRNA. The derivation of an antisense sequence, based upon a cDNA sequence encoding a target protein is described in, for example, Stein and Cohen, *Cancer Res* 48:2659 (1988) and van 15 der Krol et al., *BioTechniques* 6:958 (1988). Antisense oligonucleotides that form triplexes with a target promoter regions inhibit the activity of that promoter, see, e.g., Joseph (1997) *Nucleic Acids Res.* 25:2182-2188; Alunni-Fabbroni (1996) *Biochemistry* 35:16361-16369; Olivas (1996) *Nucleic Acids Res* 24:1758-1764. Alternatively, antisense oligonucleotides that hybridize to the promoter sequence can be used to inhibit promoter activity.

In addition to antisense polynucleotides, ribozymes can be designed to inhibit 20 expression of target molecules. A ribozyme is an RNA molecule that catalytically cleaves other RNA molecules. Accordingly, SM-MHC promoter sequences may be used to express ribozymes specifically in smooth muscle cells by linking a polynucleotide encoding a ribozyme to an SM-MHC promoter sequence. Methods for constructing and using ribozymes to treat smooth muscle 25 cancer in particular are described by Dorai et al., *Smooth muscle* 32:246-58 (1997); Norris et al., *Adv Exp Med Biol* 465:293-301 (2000). Different kinds of ribozymes have been described, including group I ribozymes, hammerhead ribozymes, hairpin ribozymes, RNase P, and axhead 30 ribozymes (see, e.g., Castanotto et al. (1994) *Adv. in Pharmacology* 25: 289-317 for a general review of the properties of different ribozymes). The general features of hairpin ribozymes are described, e.g., in Hampel et al. (1990) *Nucl. Acids Res.* 18: 299-304; Hampel et al. (1990) European Patent Publication No. 0 360 257; U.S. Patent No. 5,254,678. Methods of preparing are well known to those of skill in the art (see, e.g., Wong-Staal et al., WO 94/26877; Ojwang et al. (1993) *Proc. Natl. Acad. Sci. USA* 90: 6340-6344; Yamada et al. (1994) *Human Gene Therapy* 1:

39-45; Leavitt *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92: 699-703; Leavitt *et al.* (1994) *Human Gene Therapy* 5: 1151-120; and Yamada *et al.* (1994) *Virology* 205: 121-126).

## 2. Therapeutic proteins

5 A wide variety of therapeutic proteins may be used to treat smooth muscle diseases.

Accordingly, the SM-MHC promoter sequences of the present invention may be used to express polynucleotides encoding therapeutic proteins specifically in smooth muscle cells. Therapeutic proteins may be of prokaryotic, eukaryotic, viral, or synthetic origin. Where the therapeutic protein is not of mammalian origin, the coding sequence of the protein may be modified for maximal

10 mammalian expression according to methods known in the art (e.g., mammalian codon usage and consensus translation initiation sites).

Therapeutic proteins that can be employed in the targeted gene delivery methods of the present invention include proteins that kill the cell when expressed, such as microbial toxins (Pang, *Cancer Gene Ther* 7:991-6 (2000)) and proteins involved in apoptosis (Li *et al.*, *Cancer Res* 61:186-91 (2001); Schumacher *et al.*, *Int J Cancer* 91:159-66 (2001); Hyer *et al.*, *Mol Ther* 2:348-58 (2000); Griffith *et al.*, *J Immunol* 165:2886-94 (2000)). Smooth muscle cells can also be targeted with proteins that sensitize smooth muscle cells to therapy. Such proteins may function by converting a prodrug to an active metabolite (e.g., thymidine kinase or cytosine deaminase; for review see Aghi *et al.*, *J Gene Med* 2:148-64 (2000)), by increasing cell permeability to a

20 therapeutic agent, by restoring hormonal responsiveness, or by rendering the cell more sensitive to radiotherapy or chemotherapeutics. See, e.g., Suzuki *et al.*, *Cancer Res* 61:1276-9 (2001); Cowen *et al.*, *Clin Cancer Res* 6:4402-8 (2000); Spitzweg *et al.*, *Cancer Res* 60:6526-30 (2000); Anello *et al.*, *J Urol* 164:2173-7 (2000); Fan *et al.*, *Cancer Gene Ther* 7:1307-14 (2000); Nielsen, *Oncol Rep* 7:1191-6 (2000); Ayala *et al.*, *Hum Pathol* 31:866-70 (2000); Boland *et al.*, *Cancer Res* 60:3484-92

25 (2000). Other proteins that can be employed include proteins that inhibit proliferation or act as anti-oncogenes or tumor suppressors (Shirakawa *et al.*, *J Gene Med* 2:426-32 (2000); Tanaka *et al.*, *Oncogene* 19:5406-12 (2000); Okegawa *et al.*, *Cancer Res* 60:5031-6 (2000); Allay *et al.*, *World J Urol* 18:111-20 (2000); Steiner *et al.*, *Cancer Res* 60:4419-25 (2000)), proteins that inhibit angiogenesis (Jin *et al.*, *Cancer Gene Ther* 7:1537-42 (2000)) and proteins that induce an immune

30 response, such as cytokines or foreign antigens (Hull *et al.*, *Clin Cancer Res* 6:4101-9 (2000)). See also U.S. Patent No. 6,136,792.

## C. Delivery system for targeted gene delivery

The expression vectors of the present invention can be transfected into cells for therapeutic purposes *in vitro* and *in vivo*. These polynucleotides can be inserted into any of a number of well-known vectors for the transfection of target cells and organisms as described below. The expression vectors can be delivered *in vivo* by administration to an individual patient, typically 5 by systemic administration (e.g., intravenous, intraperitoneal, intramuscular, subdermal, or intracranial infusion) or topical application, as described below. Alternatively, vectors can be delivered to cells *ex vivo*, such as cells explanted from an individual patient (e.g., lymphocytes, bone marrow aspirates, tissue biopsy) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient, usually after selection for cells which have incorporated 10 the vector.

*Ex vivo* cell transfection for diagnostics, research, or for gene therapy (e.g., via re-infusion of the transfected cells into the host organism) is well known to those of skill in the art. In a preferred embodiment, cells are isolated from the subject organism, transfected with a polynucleotide (gene or cDNA), and re-infused back into the subject organism (e.g., patient).

15 Various cell types suitable for *ex vivo* transfection are well known to those of skill in the art (see, e.g., Freshney *et al.*, *Culture of Animal Cells, A Manual of Basic Technique* (3rd ed. 1994)) and the references cited therein for a discussion of how to isolate and culture cells from patients).

20 Vectors (e.g., retroviruses, adenoviruses, liposomes, etc.) containing therapeutic polynucleotides can also be administered directly to the organism for transduction of cells *in vivo*.

Alternatively, naked DNA can be administered. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells. Suitable methods of administering such polynucleotides are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

25 Such gene therapy procedures have been used to correct acquired and inherited genetic defects, cancer, and viral infection in a number of contexts. The ability to express artificial genes in humans facilitates the prevention and/or cure of many important human diseases, including many diseases which are not amenable to treatment by other therapies (for a review of gene therapy procedures, see Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, TIBTECH

30 11:211-217 (1993); Mitani & Caskey, TIBTECH 11:162-166 (1993); Mulligan, *Science* 926-932 (1993); Dillon, TIBTECH 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10):1149-1154 (1998); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada *et al.*, in

*Current Topics in Microbiology and Immunology* (Doerfler & Böhm eds., 1995); and Yu *et al.*, *Gene Therapy* 1:13-26 (1994)).

Delivery of the gene or genetic material into the cell is the first step in gene therapy treatment of disease. A large number of delivery methods are well known to those of skill in the art. Preferably, the polynucleotides are administered for *in vivo* or *ex vivo* gene therapy uses. Non-viral vector delivery systems include DNA plasmids, naked polynucleotide, and polynucleotide complexed with a delivery vehicle such as a liposome. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell.

Methods of non-viral delivery of polynucleotides include lipofection,

- 10 microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:polynucleotide conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA.
- 11 Lipofection is described in, *e.g.*, US Pat. No. 5,049,386, US Pat. No. 4,946,787; and US Pat. No. 4,897,355 and lipofection reagents are sold commercially (*e.g.*, Transfectam™ and Lipofectin™).
- 12 Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of
- 15 polynucleotides include those of Felgner, WO 91/17424, WO 91/16024. Delivery can be to cells (*ex vivo* administration) or target tissues (*in vivo* administration).

16 The preparation of lipid:polynucleotide complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (*see, e.g.*, Crystal, *Science* 270:404-410 (1995); Blaese *et al.*, *Cancer Gene Ther.* 2:291-297 (1995); Behr *et al.*, *Bioconjugate Chem.* 5:382-389 (1994); Remy *et al.*, *Bioconjugate Chem.* 5:647-654 (1994); Gao *et al.*, *Gene Therapy* 2:710-722 (1995); Ahmad *et al.*, *Cancer Res.* 52:4817-4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787).

20 The use of RNA or DNA viral based systems for the delivery of polynucleotides take advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (*in vivo*) or they can be used to treat cells *in vitro* and the modified cells are administered to patients (*ex vivo*). Conventional viral based systems for the delivery of polynucleotides could include retroviral, lentivirus, adenoviral, adeno-associated and herpes simplex virus vectors for gene transfer. Viral vectors are currently the most efficient and versatile method of gene transfer in target cells and tissues. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in long term expression of the

inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues.

The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral 5 vector that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system would therefore depend on the target tissue. Retroviral vectors are comprised of *cis*-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum *cis*-acting LTRs are sufficient for replication and 10 packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), simian immunodeficiency 15 virus (SIV), human immunodeficiency virus (HIV), and combinations thereof (see, e.g., Buchscher *et al.*, *J. Virol.* 66:2731-2739 (1992); Johann *et al.*, *J. Virol.* 66:1635-1640 (1992); Sommerfelt *et al.*, *Virol.* 176:58-59 (1990); Wilson *et al.*, *J. Virol.* 63:2374-2378 (1989); Miller *et al.*, *J. Virol.* 65:2220-2224 (1991); PCT/US94/05700).

In applications where transient expression of the polynucleotide is preferred, 20 adenoviral based systems are typically used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors are also used to transduce 25 cells with target polynucleotides, e.g., in the *in vitro* production of polynucleotides and peptides, and for *in vivo* and *ex vivo* gene therapy procedures (see, e.g., West *et al.*, *Virology* 160:38-47 (1987); U.S. Patent No. 4,797,368; WO 93/24641; Kotin, *Human Gene Therapy* 5:793-801 (1994); Muzyczka, *J. Clin. Invest.* 94:1351 (1994)). Construction of recombinant AAV vectors are 30 described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin *et al.*, *Mol. Cell. Biol.* 5:3251-3260 (1985); Tratschin *et al.*, *Mol. Cell. Biol.* 4:2072-2081 (1984); Hermonat & Muzyczka, *Proc. Natl. Acad. Sci. U.S.A.* 81:6466-6470 (1984); and Samulski *et al.*, *J. Virol.* 63:03822-3828 (1989).

In particular, a number of viral vector approaches are currently available for gene 35 transfer in clinical trials, with retroviral vectors by far the most frequently used system. All of these viral vectors utilize approaches that involve complementation of defective vectors by genes inserted into helper cell lines to generate the transducing agent.

pLASN and MFG-S are examples are retroviral vectors that have been used in clinical trials (Dunbar *et al.*, *Blood* 85:3048-305 (1995); Kohn *et al.*, *Nat. Med.* 1:1017-102 (1995); Malech *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 94:22 12133-12138 (1997)). PA317/pLASN was the first therapeutic vector used in a gene therapy trial. (Blaese *et al.*, *Science* 270:475-480 (1995)).

- 5 Transduction efficiencies of 50% or greater have been observed for MFG-S packaged vectors (Ellem *et al.*, *Immunol Immunother.* 44(1):10-20 (1997); Dranoff *et al.*, *Hum. Gene Ther.* 1:111-2 (1997)).

Recombinant adeno-associated virus vectors (rAAV) are a promising alternative gene delivery systems based on the defective and nonpathogenic parvovirus adeno-associated type 10 virus. All vectors are derived from a plasmid that retains only the AAV 145 bp inverted terminal repeats flanking the transgene expression cassette. Efficient gene transfer and stable transgene delivery due to integration into the genomes of the transduced cell are key features for this vector system (Wagner *et al.*, *Lancet* 351:9117 1702-3 (1998), Kearns *et al.*, *Gene Ther.* 9:748-55 (1996)).

15 Replication-deficient recombinant adenoviral vectors (Ad) are predominantly used in transient expression gene therapy, because they can be produced at high titer and they readily infect a number of different cell types. Most adenovirus vectors are engineered such that a transgene replaces the Ad E1a, E1b, and E3 genes; subsequently the replication defective vector is propagated in human 293 cells that supply deleted gene function in trans. Ad vectors can transduce multiple types of tissues *in vivo*, including nondividing, differentiated cells such as those found in 20 the liver, kidney and muscle system tissues. Conventional Ad vectors have a large carrying capacity. An example of the use of an Ad vector in a clinical trial involved polynucleotide therapy for antitumor immunization with intramuscular injection (Sterman *et al.*, *Hum. Gene Ther.* 7:1083-9 (1998)). Additional examples of the use of adenovirus vectors for gene transfer in clinical trials include Rosenecker *et al.*, *Infection* 24:1:5-10 (1996); Sterman *et al.*, *Hum. Gene Ther.* 9:7 1083-25 1089 (1998); Welsh *et al.*, *Hum. Gene Ther.* 2:205-18 (1995); Alvarez *et al.*, *Hum. Gene Ther.* 5:597-613 (1997); Topf *et al.*, *Gene Ther.* 5:507-513 (1998); Sterman *et al.*, *Hum. Gene Ther.* 7:1083-1089 (1998).

30 D. Pharmaceutical compositions

The invention provides pharmaceutical compositions that comprise SM-MHC promoter-containing therapeutic polynucleotides (*e.g.*, oligo- and poly-nucleotides, expression vectors, gene therapy constructs, etc.) alone or in combination with at least one other agent, such as, *e.g.*, a stabilizing compound, diluent, carrier, cell targeting agent, or another active ingredient or

agent. The therapeutic agents of the invention may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. Any of these molecules can be administered to a patient alone, or in combination with other agents, drugs or hormones, in pharmaceutical compositions where it is mixed with suitable excipient(s),  
5 adjuvants, and/or pharmaceutically acceptable carriers. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered (e.g., polynucleotide, protein, modulatory compounds or transduced cell), as well as by the particular method used to administer the composition. Accordingly, there are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (see, e.g., *Remington's Pharmaceutical Sciences*, 17<sup>th</sup> ed.,  
10 1989).

Pharmaceutical compositions of the invention include SM-MHC promoter-containing polynucleotides in an effective amount to achieve the intended purpose.

"Therapeutically effective amount" or "pharmacologically effective amount" are well recognized phrases and refer to that amount of an agent effective to produce the intended pharmacological  
15 result. For example, a therapeutically effective amount is an amount sufficient to treat a disease or condition or ameliorate the symptoms of the disease being treated. The therapeutically effective dose can be estimated initially either in cell culture assays or in any appropriate animal model. The animal model is also used to estimate appropriate dosage ranges and routes of administration in humans. In determining the effective amount of the vector to be administered, the physician  
20 evaluates circulating plasma levels of the vector, vector toxicities, progression of the disease, and the production of anti-vector antibodies. In general, the dose equivalent of a naked polynucleotide from a vector is from about 1  $\mu$ g to 100  $\mu$ g for a typical 70 kilogram patient, and doses of vectors which include a retroviral particle are calculated to yield an equivalent amount of therapeutic polynucleotide.

25 The pharmaceutical compositions of the invention can be administered by any means, such as, e.g., injection, oral administration, inhalation, transdermal, or parenteral application. Methods of parenteral delivery include e.g., topical, intra-arterial (e.g., directly to the tumor), intramuscular (IM), subcutaneous (SC), intramedullary, intrathecal, intraventricular, intravenous (IV), intraperitoneal (IP), or intranasal administration. Further details on techniques for  
30 formulation and administration may be found in the latest edition of "REMINGTON'S PHARMACEUTICAL SCIENCES" (Maack Publishing Co, Easton PA). See also, e.g., PCT publication WO 93/23572.

## V. Screening for Modulators of SM-MHC Promoter/Enhancer

The invention also provides constructs, cell lines and methods for screening for small molecule modulators of SM-MHC promoter/enhancer activity *in vitro* and *in vivo*. Many 5 assays are available that screen for small molecule modulators of SM-MHC transcription, including high throughput assays.

As described in detail in the Examples, results from constructs containing an SM-MHC promoter/enhancer sequence and a marker gene (in this example, the lacZ gene) indicated that various motifs of SM-MHC promoter/enhancer sequence play a role in the SM specificity and 10 subtype specificity of the SM-MHC promoters. These constructs can be employed for high throughput screening of modulators of the SM-MHC modulators. Additional *cis*-acting regulatory elements within an SMC promoter/enhancer can also be identified as described in the present invention.

The present invention also encompasses assays for identifying compounds that 15 modulate expression under the SM-MHC promoter sequences. Such modulatory compounds are useful in enhancing or inhibiting the expression of genes transcribed by the SM-MHC promoters, thus providing additional control and specificity over their expression. Compounds and other substances that modulate expression of the SM-MHC promoter/enhancer can be screened using *in* vitro cellular systems. After applying a compound or other substance to the test system, RNA can 20 be extracted from the cells. The level of transcription of a specific target gene can be detected using, for example, standard RT-PCR amplification techniques and/or Northern analysis.

Alternatively, the level of target protein production can be assayed by using antibodies that detect the target gene protein. Preferably, the SM-MHC can be fused to a reporter gene and the expression of the reporter gene can be assessed. Such reporter genes, for which assays are well 25 known to those of skill in the art, include, but are not limited to *lacZ*,  $\beta$  glucuronidase, enhanced green fluorescence protein, etc. See, e.g., Khodjakov *et al.*, 1997, *Cell. Motil. Cytoskeleton*, 38:311-317. The level of expression is compared to a control cell sample which was not exposed to the test compound. The activity of the compounds also can be assayed *in vivo* using transgenic animals according to the methods described, for example, in Examples 2-5, below.

30 Compounds that can be screened for modulation of expression of the target gene include, but are not limited to, small inorganic or organic molecules, peptides, such as peptide hormones analogs, steroid hormones, analogs of such hormones, and other proteins. Compounds that down-regulate expression include, but are not limited to, oligonucleotides that are

complementary to the 5'-end of the mRNA of the SM-MHC and inhibit transcription by forming triple helix structures, and ribozymes or antisense molecules which inhibit translation of the target gene mRNA. Techniques and strategies for designing such down-regulating test compounds are well known to those of skill in the art.

5

A. Identifying *cis*-acting elements of SM-MHC promoter/enhancer

Multiple *cis*-elements identified within the first 4.2-kb of 5'-flanking sequence of the SM-MHC promoter are critical for expression in cultured SMC. (White S.L. *et al.*, 1996, *J. Biol. Chem.*, 271:15008-15017; Katoh Y. *et al.*, 1994, *J. Biol. Chem.*, 269:30538-30545;

10 Wantanabe M. *et al.*, 1996, *Circ. Res.*, 78:978-989; Kallmeier R.C. *et al.*, 1995, *J. Biol. Chem.*, 270:30949-30957; Madsen C.S. *et al.*, 1997, *J. Biol. Chem.*, 272:6332-6340; Madsen C.S. *et al.*, 1997, *J. Biol. Chem.*, 272:29842-29851). The fact that the p4.2-*lacZ* construct was found to be active in cultured SMC, but completely inactive *in vivo*, indicates that additional regulatory elements are necessary for expression within the *in vivo* context. Furthermore, the fact that the 15 p4.2-Intron-*lacZ* construct containing approximately 16 kb of the rat SM-MHC genomic region from -4.2 kb to +11.7 kb was expressed in SMC-tissues within transgenic mice whereas the p4.2-*lacZ* construct was inactive, strongly suggests that the first 11.6 kb region of intron I contains enhancer elements required for expression *in vivo* but not in cultured SMC.

20 Additional *cis*-acting elements of SM-MHC promoter/enhancer can be identified

20 using methods of molecular genetic analysis well known in the art. For example, the location of *cis*-regulatory elements within a promoter/enhancer may be identified using methods such as DNase or chemical footprinting (e.g. Meier *et al.*, 1991, *Plant Cell* 3:309-315) or gel retardation (e.g., Weissenbom & Larson, 1992, *J. Biol. Chem.* 267:6122-6131; Beato, 1989, *Cell* 56:335-344; Johnson *et al.*, 1989, *Ann. Rev. Biochem.* 58:799-839). Additionally, resectioning experiments also 25 may be employed to define the location of the *cis*-regulatory elements. For example; a promoter/enhancer containing fragment may be resected from either the 5' or 3' end using restriction enzyme or exonuclease digests.

Another method for identifying transcriptional regulatory motifs involves modifying 30 putative *cis*-acting regulatory subsequences and assessing the change, if any, of the resultant SM-MHC promoter/enhancer to modulate transcription. The modification can be, *e.g.*, one or more residue deletions, residue substitution(s), chemical alteration(s) of nucleotides, and the like. The (modified) promoter can be operably linked to a transcribable sequence (*e.g.*, reporter genes). The relative increase or decrease the modification has on transcriptional rates can be determined, *e.g.*,

by measuring the ability of the unaltered SM-MHC promoter/enhancer to transcriptionally activate the reporter coding sequence under the same conditions as used to test the modified promoter. An increase or decrease in the ability of the modified SM-MHC promoter/enhancer to induce transcription as compared to the unmodified promoter construct identifies a *cis*-acting 5 transcriptional regulatory sequence that is involved in the modulation of SM-MHC promoter/enhancer activity.

The reporter gene can encode any detectable protein known in the art, *e.g.*, detectable by fluorescence or phosphorescence or by virtue of its possessing an enzymatic activity. In alternative embodiments, the detectable protein is firefly luciferase, alpha-glucuronidase, alpha-10 galactosidase, chloramphenicol acetyl transferase, green fluorescent protein, enhanced green fluorescent protein, and the human secreted alkaline phosphatase.

**B. Identifying SM-MHC promoter/enhancer *trans*-acting transcriptional regulatory factors**

The invention provides means to identify and isolate *trans*-acting transcriptional regulatory factors that are involved in modulating the activity of the SM-MHC promoter/enhancer. 15 Identification of *cis*-acting motifs by, *e.g.*, sequence identity comparison, can be a useful initial means to identify promoter sequences bound by *trans*-acting factors. For example, as discussed above, the hSM-MHC and rSM-MHC promoter/enhancers contain a variety of *cis*-acting motifs 20 (e.g., the CArG motifs and the G/C repressor).

After positive or tentative identification of a *cis*-acting binding site in an SM-MHC promoter/enhancer, these sequences are used to isolate the *trans*-acting transcriptional regulatory factor(s) by any means known in the art. In some embodiments, the *trans*-acting factors are isolated using sequence-specific oligonucleotide affinity chromatography, the oligonucleotides comprising 25 SM-MHC promoter sequences of the invention.

Another method tests the ability of the *cis*-acting elements to bind soluble polypeptide *trans*-acting factors isolated from different cellular compartments, particularly *trans*-acting factors expressed in nuclei. For identification and isolation of factors that stimulate transcription, cell (*e.g.* nuclear) extracts from cells that express SM-MHC are used. Means to 30 conduct these studies are well known in the art (see also Example 5).

Furthermore, as discussed further below, once a *cis*-acting motif, or element, is identified, it can be used to identify and isolate *trans*-acting factors in a variety of cells and under different conditions (*e.g.*, cell proliferation versus cell senescence). Accordingly, the invention

provides a method for screening for trans-acting factors that modulate SM-MHC promoter/enhancer activity under a variety of conditions, developmental states, and cell types (including, e.g., normal versus immortal versus malignant phenotypes).

5 C. High throughput screening of small molecule modulators of SM-MHC promoter

The invention provides constructs and methods for screening modulators, in a preferred embodiment, small molecule modulators, of SM-MHC promoter/enhancer activity *in vitro* and *in vivo*. The invention incorporates all assays available to screen for small molecule modulators of SM-MHC transcription. In a preferred embodiment, high throughput assays are adapted and used with the SM-MHC promoter/enhancer sequences and constructs provided by the invention. See, e.g., Schultz (1998) *Bioorg Med Chem Lett* 8:2409-2414; Weller (1997) *Mol Divers.* 3:61-70; Fernandes (1998) *Curr Opin Chem Biol* 2:597-603; Sittampalam (1997) *Curr Opin Chem Biol* 1:384-91.

One embodiment of the invention provides a method of screening and isolating an SM-MHC promoter/enhancer binding compound by contacting an SM-MHC promoter/enhancer sequence of the invention (particularly, an identified *cis*-acting regulatory sequence) with a test compound and measuring the ability of the test compound to bind the selected polynucleotide. The test compound, as discussed above, can be any agent capable of specifically binding to an SM-MHC promoter/enhancer activity, including compounds available in chemical (e.g., combinatorial) libraries, a cell extract, a nuclear extract, a protein or peptide.

A variety of well-known techniques can be used to identify polypeptides which specifically bind to SM-MHC promoter/enhancer sequences, e.g., mobility shift DNA-binding assays, methylation and uracil interference assays, DNase and hydroxy radical footprinting analysis, fluorescence polarization, and UV crosslinking or chemical cross-linkers. For a general overview, see, e.g., Ausubel, *supra*, (chapter 12, DNA-Protein Interactions); McLaughlin (1996) *Am. J. Hum. Genet.* 59:561-569; Tang (1996) *Biochemistry* 35:8216-8225; Lingner (1996) *Proc. Natl. Acad. Sci. USA* 93:10712; and Chodosh (1986) *Mol. Cell. Biol.* 6:4723-4733. Where an antibody may already be available or one can be easily generated, co-immunoprecipitation analysis can be used to identify and isolate SM-MHC promoter/enhancer-binding, trans-acting factors. The trans-acting factor can be characterized by peptide sequence analysis. Once identified, the function of the protein can be confirmed by methods known in the art, for example, by competition experiments, factor depletion experiments using an antibody specific for the factor, or by competition with a mutant factor.

Alternatively, SM-MHC promoter/enhancer-affinity columns can be generated to screen for potential SM-MHC binding proteins. In a variation of this assay, SM-MHC promoter/enhancer sequence or a subsequences is biotinylated, reacted with a solution suspected of containing a binding protein, and then reacted with a streptavidin affinity column to isolate the 5 polynucleotide or binding protein complex (see, e.g., Grabowski (1986) *Science* 233:1294-1299; Chodosh (1986) *supra*). The promoter-binding protein can then be conventionally eluted and isolated. Mobility shift DNA-protein binding assay using nondenaturing polyacrylamide gel electrophoresis (PAGE) is an extremely rapid and sensitive method for detecting specific 10 polypeptide binding to DNA (see, e.g., Chodosh (1986) *supra*, Carthew (1985) *Cell* 43:439-448; Trejo (1997) *J. Biol. Chem.* 272:27411-27421; Bayliss (1997) *Nucleic Acids Res.* 25:3984-3990).

Interference assays and DNase and hydroxy radical footprinting can be used to identify specific residues in the polynucleotide protein -binding site, see, e.g., Bi (1997) *J. Biol. Chem.* 272:26562-26572; Karaoglu (1991) *Nucleic Acids Res.* 19:5293-5300. Fluorescence polarization is a powerful technique for characterizing macromolecular associations and can 15 provide equilibrium determinations of protein-DNA and protein-protein interactions (see, e.g., Lundblad, 1996, *Mol. Endocrinol.* 10:607-612).

Proteins identified by these techniques can be further separated on the basis of their size, net surface charge, hydrophobicity and affinity for ligands. In addition, antibodies raised 20 against such proteins can be conjugated to column matrices and the proteins immunopurified. All of these general methods are well known in the art. See, e.g., Scopes, R. K., *Protein Purification: Principles and Practice*, 2nd ed., Springer Verlag, (1987).

The following examples are provided for illustrative purposes and are not intended to limit the scope of the invention.

## 25 EXAMPLES

### **Example 1: General Methods For Analyzing SM-MHC Transgenes**

#### **1. Isolation and cloning of the SM-MHC promoter/enhancer**

The SM-MHC gene contains a very short untranslated first exon (88 base pairs in 30 rat) that is followed by a greater than 20 kb first intron. Babij P. *et al.*, 1991, *Proc. Natl. Acad. Sci.*, 88: 10676. The cloning and sequence of the 5'-flanking region of the rat SM-MHC gene (-4229 to +88) has been previously reported. White S.L. *et al.*, 1996, *J. Biol. Chem.*, 271:15008-15017; Madsen C.S. *et al.*, 1997, *J. Biol. Chem.*, 272:6332-6340. To obtain 5'-flanking sequences with

additional intronic DNA, a rat genomic phage library (Stratagene Corp. La Jolla, CA) was screened utilizing standard Southern blotting techniques, and a  $^{32}\text{P}$ -radiolabeled 45-mer oligonucleotide corresponding to the conserved untranslated first exon as a probe (nucleotides +14 to +58). One of the positive recombinant lambda phage clones identified contained an approximately 16 kb insert 5 (determined by restriction enzyme and sequence analyses) that spanned the SM-MHC gene from -4,216 to +11,795. Identical restriction enzyme patterns between rat genomic DNA and multiple positive clones revealed that none of the clones identified had undergone rearrangement.

The nucleotide sequence of the rat clone which was used as the SM-MHC promoter/enhancer of the present invention is shown in SEQ ID N:21. The clone spans the rat 10 MHC gene from position -4,216 in relation to the transcription start site to position +11,795 downstream of the transcription start site, thus, containing about 16,011 base pairs in total.

Furthermore, since the first exon of the rat MHC gene is 88 base pairs in length, the clone extends 15 to +11,707 base pairs within the first intron.

Although the instant example describes the cloning and isolation of the rat SM-15 MHC promoter/enhancer, key regulatory regions within this polynucleotide sequence are known to be conserved across all species that express the gene. Thus, the instant invention encompasses not only the rat SM-MHC, but also the SM-MHC of other mammals, including, but not limited to, 20 humans, rabbits and mice. The full length human SM-MHC gene sequence has previously been deposited with the Institute for Genomic Research in Rockville, MD, and is assigned Ace. No. U91

20 323 and NID No. G233 5056. It can be accessed at [http://www.ncbi.nlm.nih.gov/htbin-post/Entrezlquery?db=n\\_d](http://www.ncbi.nlm.nih.gov/htbin-post/Entrezlquery?db=n_d). This sequence is hereby incorporated by reference in its entirety.

Based upon a comparison of the human and rat SM-MHC gene sequences, Figure 11 shows the 25 high degree of homology that exists between the rat and human genes. In fact, as shown in Figure 11, critical regulatory sequences are 100% conserved within the genes. Furthermore, it has previously been shown that similar regulatory sequences are conserved in the rabbit and mouse genes for SM-MHC. See, Madsen *et al.*, 1997, *J. Biol. Chem.* 272:6332.

## 2. Construction of the SM-MHC-*lacZ* reporters

To facilitate removal of pBS plasmid DNA from the pBS-*lacZ* vector, the pBS-*lacZ* 30 vector was modified by inserting Not I restriction enzyme recognition sites at the HindIII and EcoRI sites located at the borders of the pBS vector sequence. Two SM-MHC-*lacZ* reporter genes were constructed for the generation of transgenic mice. One construct (*p4.2 lacZ*) was created by ligating about a 4.3 kb BgIII fragment that extended from -4220 to +88 into a unique BamHI site of

the pBS-*lacZ* vector, and the other construct tested (*p4.2-Intron-lacZ*) was generated by subcloning an approximately 16kb SalI fragment that extended from -4229 to about +11,700 into the SalI site of the pBS-*lacZ* vector. To facilitate splicing of the *p4.2-Intron-lacZ* construct, a synthetic splice acceptor site was ligated into the KpnI site of the pBS-*lacZ* vector prior to insertion of the SM-  
5 MHC DNA fragment. The location of the KpnI site, between the SalI site and the *lacZ*-gene, allowed for the correct positioning of the splice acceptor site at the +11,700 end of the SM-MHC intron. The proper construction of each SM-MHC-*lacZ* chimeric plasmid was verified by sequencing and restriction enzyme analyses. As an additional precaution against cloning artifacts, both transgenic constructs were tested for *lacZ* expression in transient transfection assays in  
10 cultured rat aortic SMC using a method that was previously described. Madsen C.S. *et al.*, 1997, *J. Biol. Chem.*, 272:6332-634

### 3. Generation and analysis of transgenic mice

Plasmid constructs *p4.2-lacZ* and *p4.2-Intron-lacZ* were tested for SM-MHC  
15 promoter activity in transgenic mice following removal of the pBS vector DNA through NotI digestion and subsequent agarose gel purification. Transgenic mice were generated using standard methods (Li L. *et al.*, 1996, *J. Cell. Biol.*, 132:849-859; Gordon J.W. *et al.*, 1981, *Science*, 2  
14:1244-1246) either commercially (DNX, Princeton, NJ) or within the Transgenic Core Facility at  
14 The University of Virginia. Transgenic mice were either sacrificed and analyzed during  
20 embryological development (transient transgenics), or were utilized to establish breeding-founder lines (stable transgenics). Transgene presence was assayed by the polymerase chain reaction using genomic DNA purified from either placental tissue (embryonic mice) or from tail clips (adult mice) according to the method of Vemet M. *et al.*, 1993, *Methods Enzymol.* 225:434-451. Transgene expression and histological analyses were done as described previously. Li L. *et al.*, 1996, *J. Cell.  
25 Biol.*, 132:849-859; Cheng T.C. *et al.*, 1993, *Science*, 261:215-218. In order to determine possible positional effects of transgene insertional sites on transgene expression, multiple independent founder lines were analyzed for each transgene construct.

### 4. SM-MHC immunohistochemistry

30 Various smooth muscle containing tissues were collected from 5-6 week old transgenic mice and fixed overnight in methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid). Tissues were subsequently dehydrated through a graded series of methanol dilutions. Fixed, dehydrated tissues were prepared for paraffin embedding by incubation in 100% xylene.

Tissue was then infiltrated by incubation through a series of xylene:paraffin(3:1, 1:1, 1:3) solutions, and two final incubations in 100% paraffin prior to embedding in 100% paraffin. Serial sections (6  $\mu$ m) were placed on uncoated slides, and then dried for approximately 45 minutes on a slide warmer set at 40°C. Sections were cleared in multiple washes of 100% xylene, and re-hydrated 5 through a graded ethanol series to a final incubation in phosphate buffered saline (PBS). Endogenous peroxidase activity was quenched by incubating slides in methanol containing 0.3% hydrogen peroxide for 30 min. Slides were subsequently rehydrated in PBS and blocked in a 1:50 solution of normal goat serum made up in PBS. Sections were then incubated with the primary antibody for 1 hr and washed with 3 changes of PBS. Detection of primary antibody was 10 performed using a Vectastain ABC Kit according to the instructions of the manufacturer with diaminobenzidine (DAB) as the chromagen (Vector Laboratories, Burlingame, CA).

#### Antibodies:

Several different SM-MHC antibodies were employed. These included a 15 monoclonal antibody designated 9A9 which has been previously characterized (Price R.J. *et al.*, 1994, *Circ. Res.*, 75:520-527) that shows reactivity with the SM-1 and SM-2 isoforms of SM-MHC but which shows no reactivity with non-muscle myosin heavy chains or other proteins. However, whereas this antibody showed some reactivity with mouse SM-MHC isoforms in Western analyses, it reacted very poorly with mouse SM-MHC in fixed tissues. In addition, although a polyclonal 20 SM-MHC peptide antibody provided by Nagai R. *et al.*, 1989, *J. Biol. Chem.*, 264:9734-9737, showed complete specificity for SM-MHC isoforms in Western analyses of smooth muscle tissues from multiple species, it showed little or no reactivity with mouse SM-MHC isoforms. To circumvent these limitations, a rabbit anti-chicken gizzard SM-MHC polyclonal antibody was employed. The rabbit anti-chicken gizzard SM-MHC antibody was made by immunization of 25 rabbits with partially purified gizzard SM-MHC as described by Groschel-Stewart, 1976, *Histochemistry* 46:229-236. However, based on Western analyses, it was determined that this antibody showed reactivity with both SM-1 and SM-2 MHC, as well as with non-muscle myosin B (or SMEMB), as did a number of other "smooth muscle myosin" antibodies tested, including one from Sigma [designated hSM-V] (Frid M.G. *et al.*, 1993, *J. Vasc. Res.*, 30:279-292) and one from 30 R.S. Adelstein (Schneider M.D. *et al.*, 1985, *J. Cell. Biol.*, 101:66). As such, staining with these antibodies in tissues that express both SMEMB and SM-MHC is equivocal. However, adult mouse aortic SMC, like those in other species (Rovner A.S. *et al.*, 1986, *J. Biol. Chem.*, 261: 14740-

14745; Rovner A.S. *et al.*, 1986, *Am. J. Physiol.*, 250:c861-c870; Phillips C.L. *et al.*, 1995, *J. Muscle Res. & Cell Motility*, 16:379-389) were not found to express SMEMB based on Western analyses. The rabbit anti-chicken gizzard SM-MHC polyclonal antibody was used at a concentration of approximately 20 µg/ml in PBS. Biotinylated goat anti-rabbit secondary antibodies were purchased from Vector Laboratories (Burlingame, CA) and used at a concentration of 10 µg/ml in PBS. Appropriate Western analyses, and immunohistological controls were performed to assess specificity, including exclusion of primary antibody, and use of control non-immune rabbit serum.

10 5. Chromatin immunoprecipitation

L6 rat skeletal myoblasts were cultured in  $\alpha$ -minimal essential medium (Lifetechnologies) supplemented with 2% FBS for 7 days to induce myotube formation. L6 myotubes, L6 myoblasts, Rat1 fibroblasts, and rat aortic SMCs in 100 mm dished were fixed directly by adding 280 µl of 37% formaldehyde to 10ml of culture media and incubating at 37°C for 10 min. The fixed cells were harvested and prepared for immunoprecipitation using the protocol of ChIP assay kit (Upstate Biotechnology) with minor modifications. A quarter of the sample was precleared with salmon sperm DNA/protein A agarose (Upstate) and subsequently incubated with either 2 µl of anti-SRF antibody (Santa Cruz Biotechnology) or no antibody at 4°C over night. Chromatin samples were immunoprecipitated using salmon sperm DNA/protein A (Upstate). Samples were washed two times with 1 ml of wash buffer A (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150 mM NaCl), once with wash buffer B (0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and two times with TE. Immune complexes were eluted and subsequently reverse-crosslinked and purified by phenol/chloroform extraction. Ethanol precipitated DNA pellets were redissolved in 40 µl of TE buffer. The supernatant of an immunoprecipitation reaction done in the absence of SRF antibody was purified and used as a control to show total input DNA. The supernatant DNA was diluted 1:100 prior to PCR. One µl of each sample was subjected to PCR amplification. PCR analysis was carried out using primers from different regions of the SM-MHC gene and promoter regions of a number of control genes that are silent in SMCs (skeletal  $\alpha$ -actin, insulin and  $\beta$ -globin). The sequences of PCR primers are shown in table 1. Following 32 (all primer sets except insulin) or 35 cycles (insulin) of amplification, PCR products were run on 2% agarose gels and analyzed by GelStar (BMA) staining. As additional controls, the promoter regions of the genes

either silent or lacking CArG elements were also amplified by PCR. The PCR samples of these promoters showed a low level of background chromatin immunoprecipitation. The sequences of the PCR primers were the following;

5 insulin, 5'-GCCAAACTCTAGGGACTT TAGGAAGGATG-3' (SEQ ID NO:10), 5'-  
GCCGGGCAACCTCCAGTGCCAAGGTCTGAAGATC-3' (SEQ ID NO:11);  $\beta$ -globin, 5'-  
CAGCGTTTCTTCAGAGGGAGTACCCAGAG-3' (SEQ ID NO:12), 5'-  
TCAGAACAAATGTGAGGAGCGACTGATCC-3' (SEQ ID NO:13); skeletal  $\alpha$ -actin, 5'-  
CAGGCTGAGAAGCAGCCGAAGGGACTCTAG-3' (SEQ ID NO:14), 5'-  
10 ACCTCCACCCCTACCTGCTGCTACTCTG-3' (SEQ ID NO:15); SM-MHC -4000, 5'-  
ATGTCAGATGTCCTCTCACTGCTTATTCC-3' (SEQ ID NO:21), 5'-  
15 ACACAAACAGCTTAAATACGTATTGGCTTC-3' (SEQ ID NO:22); 5'-CArG, 5'-  
CTGGAGCTCTATTAGTACTGGGGTCCC-3' (SEQ ID NO:18), 5'-  
ACTCAGGCCATAAAAGGAAGTCGAGGCAGAGTTGG-3' (SEQ ID NO:19); intronic CArG,  
20 5'-GGC CAAGCCACCCCTGGAGAACCTGGAC-3' (SEQ ID NO:20), 5'-  
CCCAGAACTCAAGCCAGTCAGGCTGCATCG-3' (SEQ ID NO:23). Due to the relatively low  
resolution of ChIP method, we designed the PCR primers for the 5'-flanking CArG region for  
amplification of the region containing both CArG1 and CArG2.

## 20 Example 2: SMC-Specific Expression in Transgenic Mice: Indispensable Elements in SM-MHC First Intron

It has previously been reported that an SM-MHC promoter DNA fragment extending from -4220 to +88 was capable of directing high-level expression in cultured rat aortic SMC. Madsen C.S. *et al.*, 1997, *J. Biol. Chem.*, 272:6332-6340. When tested in bovine endothelial cells, 25 L6 myoblasts and L6 myotubes, the activity of this construct was determined to be negligible. To determine if this same promoter/DNA fragment was capable of directing SMC-specific expression *in vivo*, this fragment was sub-cloned into a pBS-*lacZ* reporter gene construct (p4.2-*lacZ*) and tested for activity in transgenic mice. Thirteen independent transient transgenic mice harboring the p4.2-*lacZ* transgene were generated and analyzed for *lacZ* expression at multiple embryological 30 stages ranging from embryonic day ("E") 13.5 to 19.5. No transgene expression was detected in any of the transgenic mice. These data show that, in contrast to activity levels observed for cultured SMC, the SM-MHC promoter fragment present within the p4.2-*lacZ* construct did not contain sufficient DNA for directing SMC-specific expression in transgenic mice.

It is well documented that *cis*-elements important for gene expression can be found outside the 5'-flanking region. Furthermore, they can be found within intronic regions. Because 4.2 kb of 5'-flanking DNA was found to be insufficient for expression *in vivo*, a larger construct with added intronic sequences was tested. A rat genomic phage library was screened and one  
5 recombinant clone was identified whose insert contained 4216 bp of 5'flanking region, 88bp of the first exon, which is untranslated sequence, and an additional 11,795 base pairs of first intronic sequence (total span: -4,216 to +11,795). This fragment, which was essentially identical to the p4.2-*lacZ* construct with respect to the 5'-flanking sequence and with respect to the presence of the 88 bp of 5' untranslated sequence, was isolated from the lambda phage by SalI digestion and sub-  
10 cloned into the pBS-*lacZ* vector to create the SM-MHC-reporter gene plasmid p4.2-Intron-*lacZ*.

The reporter gene p4.2-Intron-*lacZ* was used to generate four independent transgenic mice; one mouse was sacrificed at E13.5 for transgene expression analysis, and the other three were established as stable transgenic founder lines (designated as 2282, 2642 and 2820) that were utilized for analysis of transgene expression throughout embryological development and early  
15 adulthood. Analysis of adult mice generated from the three stable founder lines showed that *lacZ* transgene expression was essentially identical between the three founders and completely restricted to smooth muscle (Figures 1, 2 and 12). Gross examination of the heart and lung region excised from a 5 week-old p4.2-Intron-*lacZ* mouse revealed that transgene expression was present in the descending thoracic aorta, coronary arteries, trachea and bronchi (Figure 12, Panel A). Transgene  
20 expression was not detected in any non-smooth muscle tissues in this region, such as heart muscle and lung tissue. Of note, transgene expression also was not detected in several smooth muscle containing tissues in this region including the esophagus and branches of the pulmonary artery, although expression was seen in the pulmonary artery outflow tract. Transgene expression was readily detectable in the major branches of the coronary arterial tree including the left and right  
25 coronary arteries (Figure 12, Panel B), as well as the small coronary arteries and arterioles (Figure 12, Panel D) of 5-6 week old transgenic mice. However, no *lacZ* expression could be detected in any of the coronary veins (Figure 12, Panels B and D; and Figure 13, Panel C). Transgene expression also was readily detected in the descending thoracic aorta, and intercostal arteries  
30 (Figure 12, Panel C), as well as throughout blood vessels in the extremities and main body trunk, including small arteries, arterioles and veins such as the mesentery vessels (Figure 12, Panel E). Expression of the *lacZ* transgene was readily detectable also in the visceral smooth muscle of the intestine (Figure 12, Panel F), the ureter and bladder (Figure 12, Panel G), the stomach (Figure 12, Panel H) and the uterus and gallbladder. Thus, these initial analyses demonstrated that the p4.2-

Intron-*lacZ* construct contained sufficient DNA for expression in all SMC tissue types, although certain SMC tissues were negative, at least in 5-6 week old animals. Moreover, certain smooth muscle tissues such as the aorta (Figure 12, Panel C), intercostal arteries (Figure 12, Panel C), jejunum (Figure 12, Panel F) and stomach (Figure 12, Panel H) clearly showed a mosaic pattern of 5 transgene expression that was visible even at the gross tissue level.

To assess transgene expression at the cellular level, histological analyses of *lacZ* reporter expressions were performed (see, e.g., Figures 3 and 13). Results of these studies further demonstrated that transgene expression was highly restrictive to smooth muscle. For example, analysis of the bladder and airway smooth muscle (Figure 13, Panel A) showed that transgene

10 expression was highly specific and appeared to be present in virtually all SMC located within these tissues. Likewise, SMC within many smooth muscle tissues including the aorta (Figure 13, Panel B), coronary vessels (Figure 13, Panel C), the intestine (Figure 13, Panel D), stomach and many 15 smaller blood vessels including small arteries, arterioles, veins, and venules (Figure 13, Panels E and F) showed clear evidence of expression of the transgene within SMC, although some heterogeneity of expression was evident between adjacent cells.

Taken together, these results indicate that although the p42-Intron-*lacZ* transgene exhibited SMC-specific activity and was expressed in all major SMC types, it exhibited differences in activity in subsets of SMC both within and between different adult SMC tissues. Nevertheless, expression of the p4.2-Intron-*lacZ* transgene was present only in SMC, and not in any non-SMC.

20

### **Example 3: Transgene Expression in the Developing Embryo**

To determine if expression of the p4.2-Intron-*lacZ* transgene resembled the developmental expression pattern of the endogenous SM-MHC gene, embryos from the three stable founder lines were obtained at various stages throughout development [embryonic day E10.5 25 through E19.5] and analyzed for *lacZ* expression. Additionally, one transient founder was generated and analyzed for transgene expression at E13.5. With the exception of transient expression in the heart (B 12.5 to E17.5) of one of the stable lines which was localized to the myocardium, transgene expression patterns were essentially identical in all four independent transgenic lines (i.e. one transient transgenic mouse and three stable founder lines), and restricted to 30 SMC. Transgene expression patterns of embryos derived from stable founder lines 2282, 2642 and 2820 are presented in Figures 14 and 15. The earliest developmental stage at which transgene expression could be detected was E12.5, where *lacZ* expression was readily identified in the trachea and bronchi (Figure 14, Panels A and B). By E14.5, transgene expression was detectable in the

bronchi, intestine, stomach, trachea and the aorta as well as a few other vessels throughout the embryo (Figure 14, Panel C). Of particular interest, although transgene expression was virtually absent in the esophagus in the adult (Figure 12, Panel H), its expression was clearly evident in embryos. At E16.5 transgene expression was more pronounced in the aorta than at earlier 5 developmental time points, although it had a variegated and less intense appearance relative to other smooth muscle tissues (Figure 14, Panel D). Additionally, the frequency of vessels that were positive for transgene expression was higher in peripheral vessels, and particularly those located in the extremities of the animal.

One of the most notable differences between the E16.5 and E19.5 embryos was a 10 marked increase in the frequency of vessels that stained positive for *lacZ* expression (Figure 15). However, *lacZ* expression remained undetectable in a number of vessels. Especially conspicuous was the general absence of expression in the large blood vessels in the head and neck region including the internal and external carotid arteries, the jugular vein and the cerebral arteries and veins. However, many smaller sized blood vessels were positive for transgene expression in the 15 head and neck region. Transgene expression was readily detectable also in many other arteries and veins throughout the body including the iliacs (Figure 15, Panel D), the caudal artery and vein, the femoral artery, the umbilical artery and vein, the ulnar and radial arteries and superficial arterioles and venules within the musculature of the thoracic cage (Figure 15).

Although expression levels in these types of studies are not quantitative, it is worth 20 noting that levels of *lacZ* staining within the aorta did not appear to be as intense as compared to many other blood vessels and visceral smooth muscle tissues. In summary, results of these embryological studies support the data gathered from analysis of transgene expression in juvenile and adult mice, and show that p4.2-Intron-*lacZ* contains sufficient DNA for directing SMC-specific expression in all SMC-tissue types. However, results leave open the possibility that additional 25 genomic regions may be required for SM-MFIC expression in some subsets of SMC. Nevertheless, these results demonstrate that the p4.2 Intron-*lacZ* transgene is capable of conferring SMC-specific gene expression *in vivo*.

#### **Example 4: Multiple CArG Elements Define SM-subtype Specificity of SM-MHC In Vivo**

##### **30 1. Plasmids construction and transfection**

Mutant transgenic constructs of SM-MHC CArG elements were made in the context of -4200 to + 11600 promoter/intron *LacZ* transgene (SM-MHC 4.2+intron- *LacZ* plasmid; Madsen et al., Circ. Res. 82:908-17, 1998). For simplicity, this construct is referred to as SM-MHC -

4200/+11600 *LacZ* in this paper. Site-directed mutagenesis was performed on small fragments subcloned in pBluescript II using GeneEditor (Stratagene). The integrity of mutated fragments was confirmed by sequencing, and the fragments were subcloned back into the parental plasmid. The resultant mutant transgenic plasmids were tested for integrity by sequencing and restriction enzyme mapping. To minimize the possibility of errors in DNA amplification, at least two independently constructed clones were tested for activity in cultured SMCs. Mutant sequences are the following: 5 CArG1, ttCCTTTATGG (SEQ ID NO:1) to ggATCCTATGG (SEQ ID NO:2); CArG2, CCTTTTG<sub>3</sub>GG (SEQ ID NO:3) to ATCCTTGGG (SEQ ID NO:4); intronic CArG, CCTTGTATGG (SEQ ID NO:5) to AGGCCTATGG (SEQ ID NO:6).

10 The minimal thymidine kinase promoter taken from pBLCAT5 (Boshart et al., Gene 110:129-30, 1992) was subcloned into pAUG *LacZ*. Subsequently a *Bst*XI/*Bgl*II (+1447 to +1673) fragment of the SM-MHC first intron was subcloned into the TK *LacZ* vector so that the fragment was repeated three times upstream of the TK promoter (3xICR-TK *LacZ*).  
15

Transfection of the plasmids was performed using DOTAP (Roche) as described 15 previously (Madsen et al., J. Biol. Chem. 272:6332-40, 1997). At least two independent clones were used for transfection and the transfection of each plasmid was done at least in duplicate. Reporter activity was assayed by using ONPG as a substrate (Manabe et al., Biochem. Biophys. Res. Commun. 239:598-605, 1997). The activity was normalized to the protein concentration of each cell lysate as measured by DC protein assay kit (BioRad). The endogenous  $\beta$ -galactosidase 20 activity was determined by transfecting a nonfunctional DNA (pBluescript II) and was subtracted from the measured activity of each construct. Subsequently the activity was normalized to that of promoterless construct, pAUG *LacZ*. One-way analysis of variance followed by Bonferroni method was used for data analysis. Values of  $p < 0.05$  were considered statistically significant.

Transgenic mice were used to establish breeding founder lines. Transgenic mice 25 were generated and analyzed as described above in Example 1. To determine possible positional effects of transgene insertional sites on transgene expression, multiple independent founder lines were analyzed for each transgene construct.

## 2. Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSA)

30 Transgenic mice were used to establish breeding founder lines. Transgenic mice were generated and analyzed as described above in Example 1. To determine possible positional

effects of transgene insertional sites on transgene expression, multiple independent founder lines were analyzed for each transgene construct.

Preparation of nuclear extracts from cultured SMCs was performed as described in Madsen (1997). Nuclear extracts from rat tissues were prepared as described previously (Dignam et al., Nucleic Acids Res. 11:1475-89, 1983) with the following modifications. In brief, tissues were taken from male Sprague-Dawley rats. Non-SMC layers were removed from the aorta, stomach, and bladder and tissues were immediately frozen in liquid nitrogen. The frozen tissues were powdered and washed once with modified buffer A (10 mM HEPES, pH 7.9, 13 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 0.05% NP-40) with Complete EDTA-free protease inhibitor (Roche).

The samples were resuspended in 10 ml of buffer A and incubated on ice for 5 min. The samples were centrifuged and resuspended in a 10 packed cell volume of buffer A. NP-40 was added to the final concentration of 0.3%. The samples were then homogenized using a Dounce homogenizer. Disruption of cell membranes was confirmed by microscopic observation. The centrifuged samples were resuspended in modified buffer C (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.01% NP-40, Complete EDTA-free) and incubated on ice with gentle agitation for 30 min. Cell debris was removed by centrifugation. The samples were changed for buffer and enriched using Ultrafree-4 concentrator (Millipore).

The sequences of sense strands of EMSA probes were the following; CArG1, 5'-gacttcctttatggctcta-3' (SEQ ID NO:7); CArG2, 5'-cctggccctttgggttgtt-3' (SEQ ID NO:8); intronic CArG, 5'-catgccttgcgtatggtagtgc-3' (SEQ ID NO:9); EMSAs were performed as described in Manabe et al., *supra*. In brief, 20 kcpm of <sup>32</sup>P-labeled probe was incubated with nuclear extracts in 20  $\mu$ l of binding buffer (10 mM Tris-HCl (pH7.5), 50 mM NaCl, 0.5 mM DTT, 10% glycerol, and 0.05% NP-40) with 0.25  $\mu$ g of poly (dI-dC) (dI-dC). Reactions were incubated on ice for 20 min. For supershift assays, 1  $\mu$ l of anti-SRF antibody was added after the 20-min incubation period and the reactions were incubated for an additional 10 min. The reactions were run on 5% polyacrylamide gels.

3. The SM-MHC contains conserved CArG elements required for maximal promoter activity in cultured SMCs

We first examined transcriptional activity of the first intron in cultured rat aortic SMCs by using a series of 3'-deletion constructs as a means to identify putative *cis*-regulatory elements that would subsequently be tested *in vivo* in transgenic mice. Deletion of the region from

+2491 to +417 decreased activity significantly (56-fold vs. 17-fold activity over pAUG *LacZ*). To identify possible *cis*-elements in the region +2491 to +417, we further analyzed this region using finer deletion mutants. A series of finer deletion mutants was constructed in the context of 1346 bp of the 5'-flanking region and transfected into cultured SMCs (Fig. 18A). Significant reductions in reporter activity were observed when the sequence from +1617 to +1586 was deleted (Fig. 18A). Within this region there is a CArG-like element at +1599 that is also present at an equivalent region in the human SM-MHC intron (Fig. 18B).

We have previously identified two CArG elements in the 5'-flanking sequence of the SM-MHC gene that are functional in the context of -1346 to +88 region in cultured SMCs (Madsen et al., J. Biol. Chem. 272:29842-51, 1997; and Madsen et al., J. Biol. Chem. 272:6332-40, 1997). However, since the 5'-flanking sequence alone is completely inactive in SMC *in vivo* in transgenic mice, it is critical to re-test the functionality of these *cis*-elements within the context of the -4200/+11600 *LacZ* construct previously shown to be active in SMCs *in vivo* (Madsen et al., 1998, *supra*). To ensure the efficacy of mutations in abrogating transcription factor binding, we first performed a series of EMSA experiments with each CArG element. Consistent with our previous results, both CArG1 and CArG2 probes bound SRF in nuclear extracts prepared from cultured SMCs (Fig. 19, lanes 21 and 22). In addition, as expected based on sequence analysis, the SM-MHC intronic CArG also exhibited SRF binding (Fig. 19, lanes 23, complexes A and B). Mutations of each CArG element completely abolished SRF binding activity in EMASAs. We then tested the effects of these same CArG mutations on transcriptional activity of the -4200/+11600 *LacZ* SM-MHC construct in cultured SMCs. The mutations of CArG1, CArG2, and intronic CArG reduced reporter activity by 46%, 49%, and 74%, respectively.

#### 4. The intronic CArG confers SM-type-selective transcription *in vivo*

The data of transgenic mouse experiments clearly demonstrated that the intronic CArG element was necessary for transcription of the SM-MHC gene in the large arteries *in vivo*. As shown in Fig. 18B, we found that the region containing the intronic CArG is highly conserved between the rat and human genes. To test if the intronic region could work as a distinct transcriptional regulatory module *in vivo*, three copies of the 227-bp sequence containing the highly homologous region (+1447 to +1673) were cloned in tandem 5' to a minimal thymidine kinase (TK) promoter *LacZ* construct (3xICR-TK *LacZ*). The construct showed very high activity (13.7-fold activity over the minimal TK *LacZ* construct) in cultured SMCs. This construct was used to produce transgenic mice. In one founder line (line 7240) among four founder lines, very strong

reporter expression was observed in vascular SMCs (see, Figure 25). Another line (line 7249) also showed expression in SMCs although not as strong. The other two lines were negative for staining. Although two founder lines did not express the 3xICR-TK *LacZ* transgene; it seems unlikely that the cell restricted activity observed in lines 7240 and 7249 was due solely to locus dependent 5 activation associate with the site of transgene insertion. Rather results support that the 227-bp intronic sequence can direct transcription at least in some SMCs *in vivo* when coupled with a minimal TK promoter.

In line 7240, reporter expression was particularly prominent in the large arteries including the aorta, carotid, and pulmonary arteries (Fig. 25, Panels A, B, F, G). Reporter 10 expression was also strong in intermediate size arteries (Fig. 25, F). Transgene expression in smaller arteries was relatively weaker than that in large arteries and not all the smaller arteries were stained positive. Reporter expression was also observed in large veins including the vena cava (Fig. 25, F). While the expression in vascular SMCs was very strong, transgene expression was very weak in visceral SMCs. Only few cells were stained positive in the stomach, intestine, and 15 bladder (Fig. 25, C-E). Interestingly, strong reporter expression was also observed in the heart and skeletal muscle. In the heart, while cardiac muscle cells were stained positive for  $\beta$ -galactosidase expression, no transgene expression was observed in SMCs in coronary vessels (Fig. 25, H). Various skeletal muscle cells also expressed the transgene (Fig. 25, I). The data provide evidence 20 that the conserved region containing the intronic CArG is capable of driving transcription in subsets of SMCs *in vivo* but lacks the complete SMC-specificity seen with the endogenous SM-MHC gene and the -4200/+11600 *LacZ* transgene.

5. Differential requirements of the CArG elements in SMC-subtypes *in vivo*  
In order to examine the functional roles of the CArG elements *in vivo*, the -  
25 4200/+11600 *LacZ* SM-MHC CArG mutant constructs were used to generate transgenic mice. The expression patterns observed are summarized in Table 1. Mutation of CArG1 resulted in abrogation of *LacZ* expression in all SM tissues in all three independent transgenic founder lines analyzed. In contrast, all three transgenic founder lines of the wild-type SM-MHC -4200/+11600 *LacZ* construct showed reporter expression in virtually all SMC tissues (Fig. 20). These data clearly demonstrate 30 that CArG1 was required for expression of the SM-MHC gene *in vivo* in all SM tissues.  
Mutation of CArG2 resulted in differential reductions in reporter activity in SM tissues. *LacZ* expression in the gastrointestinal (GI) tract was decreased but was easily detectable

in adult mice (Fig. 20, I, M vs. K, O). Expression in the bladder was similar to that observed in wild-type mice (Fig. 20, Q vs. S). No expression was observed in large blood vessels including the aorta, pulmonary, coronary, carotid, celiac, and femoral arteries, and the vena cava (Fig. 20, A, E vs. C, G). However, very weak reporter expression was observed in smaller arteries including 5 small mesenteric arteries (data not shown). Mutation of CArG2 also virtually abolished expression in the trachea and bronchi (Fig. 20, E vs. G).

10 **Table 1 Summary of report gene expression in SM-MHC *LacZ* transgenic mice**

construct	<i>LacZ</i> positive lines/ founder lines	aorta	coronary artery	mesenteric artery	vena cava	airways	stomach	intestine	bladder
-4200/+11600 <i>LacZ</i>	3/3	++	++	++ -	++	++	++	++	++
CArG1 mutant	0/3	-	-	-	-	-	-	-	-
CArG2 mutant	3/5	-	-	±	-	±	+	+	+
intronic CArG mutant	4/4	-	+	++	++	++	++	++	++
3xICR-TK <i>LacZ</i>	2/4	+++	-	++	++	±	±	±	±

15 Mutation of the intronic CArG resulted in a vascular SMC-specific phenotype.

Reporter expression in the GI tract, urinary tract and airways was equivalent to that of wild-type transgenic mice in adults and embryos of four independent intronic CArG mutant lines (Fig. 20, Panels B, I, M, Q vs. H, L, P, T). Expression in veins was also equivalent to that of the wild-type. However, expression in large arteries including the aorta, common carotid arteries and the main 20 trunks of subclavian arteries was completely silenced in all lines (Fig. 20, A, E vs. D, H).

Interestingly, the small branching arteries from the thoracic aorta including the intercostal arteries showed transgene expression equivalent to that of the wild-type (Fig. 21, C, D). In the carotid arteries no expression was observed in the proximal portion (Fig. 21, E), whereas in the distal common carotid arteries a few cells were stained positive, and the internal and external carotid 25 arteries were strongly stained. Strong expression of the intronic CArG mutant was also observed in arteries in the head including the basilar artery, arteries of Willis ring, and cerebral arteries (data

not shown). Reporter expression was not detected in the abdominal aorta, whereas the branching arteries from the abdominal aorta including the celiac, renal, and adrenal arteries were stained strongly positive (Fig. 21, A vs. B). Indeed, the abrupt transition in expression from non-detectable to a high level between the conduit arteries and branch arteries was quite remarkable (Fig. 21, B, 5 F). Histological sectioning of blood vessels in the abdomen clearly showed selective abrogation of reporter expression in the aorta in intronic CArG mutant transgenic mice (Fig. 21, G- J). In the common iliac arteries expression was barely detectable, whereas expression was strong in their branches including the femoral arteries (Fig. 21, F).

Transgene expression in the coronary arteries was somewhat varied among the 10 intronic CArG mutant lines presumably due to positional effects of transgene insertion sites. In two lines some expression was detectable in the coronary arteries, while little or no expression was observed in the other lines. However, even in the former two lines, overall transgene expression was clearly much weaker than that of the wild-type transgenic lines. Positive staining was restricted within the main trunks and a few major branches in the intronic CArG mutants, while in 15 the wild-type expression was detectable in smaller branches. However, due to the qualitative nature of  $\beta$ -galactosidase staining and variability in expression level among the lines we could not conclude the extent of necessity of the intronic CArG in the coronary arteries.

Similarly, expression in the pulmonary arteries and veins was varied in mice 20 containing the mutant intronic CArG transgene. Two lines, which showed transgene expression in the coronary arteries, had detectable transgene expression in the pulmonary blood vessels, whereas the other lines showed no expression. Even in two expressing lines, transgene expression was very weak, which made the staining of the lung look sparse as compared with that of the lung of wild-type mice as depicted in Fig. 20. However, microscopically some SMCs in the pulmonary blood vessels were stained positive (data not shown). Expression of the wild-type transgene in the 25 pulmonary vessels was also somewhat varied among transgenic lines and the expression level, especially that in the pulmonary veins, was generally weak as compared with other vascular beds. Given the variability and weakness of transgene expression in the pulmonary circulation, no definite conclusions can be made regarding the role of the intronic CArG in pulmonary blood vessels, although results showing no expression in two founder lines suggests that it may have 30 some function. Reporter expression in intronic CArG mutant transgenic mice showed that the intronic CArG was indispensable for transgene expression in large arteries, while it was

dispensable in smaller arteries, veins and visceral SMCs. The large arteries that absolutely required the intronic CArG largely fit the classification of elastic artery.

The SM-MHC gene is a marker of later stage SM differentiation, and expression of the wild-type -4200/+11600 *LacZ* transgene was relatively weak in many SMC tissues until 5 embryonic day (ED) 17.5-19.5. Thus, to facilitate analysis of effects of CArG mutations during development, we restricted analyses to ED 19.5. Results showed the transgene expression pattern in each CArG mutant transgenic mouse was largely consistent with that in adult mice (Fig. 22). No expression was observed in embryos of CArG1 mutant transgenic mice. In the CArG2 mutant transgenic mice, reporter expression was observed only in the GI tract. In intronic CArG mutant 10 transgenic mice, the expression in the GI tract and airways was equivalent to that in the wild-type transgenic lines. While reporter expression in smaller arteries was easily detectable, no expression 15 was detected in the large arteries in the intronic CArG mutant transgenic mouse embryos.

In summary, the transgenic mouse data demonstrate that each CArG element is differentially required in SMC-subtypes *in vivo* in transgenic mice. CArG1 is crucial for 20 transcription in all SMCs; CArG2 is indispensable in large blood vessels but had a relatively minor role in the GI and urinary tracts; the intronic CArG is absolutely required only in large elastic arteries. Taken together, the results demonstrate the multiplicity of regulatory programs that control expression of SMC differentiation marker genes *in vivo* and indicate that each of the 25 multiple CArG elements mediates distinct information for transcriptional regulation in different cell-types *in vivo*. In addition, the data indicate that the spatial and temporal regulation of SMC genes is not governed by a single regulatory region or an enhancer.

## 6. Discussions

The results of the transgenic mice of the CArG mutant constructs indicate that at 25 least two regions (i.e., the 5'-flanking CArG and intronic CArG regions) are required for *in vivo* transcription of the SM-MHC gene. We are further mapping transcriptional regulatory modules in the SM-MHC gene locus. Preliminary data indicate that the 5'-flanking and first intron contain multiple positive and negative transcriptional regulatory regions, and that different SMC-subtypes require different subsets of modules (Manabe and Owens, unpublished observations). Why might 30 SM-MHC transcription require such a complex transcriptional regulatory scheme *in vivo*?

It is evident that vascular SMCs within different vascular beds reside in vastly divergent local environments *in vivo*. Differences in the physiological role of vascular beds with respect to blood pressure, flow, and tone require very diverse vessel wall structures. SMCs are thus

undoubtedly exposed to quite different vasoactive/neuronal stimuli and environmental cues from one vascular bed to another. If one considers the differences between vascular and visceral SMCs, the diversity is even more prominent, and it is well established that SMCs are derived from different embryological origins. Finally, one must also consider that the functions of SMCs can 5 vary greatly during development and in adult animals due to their key role in matrix deposition, and vessel morphogenesis, as well as in vascular repair. Due to these many differences, SMCs *in vivo* need to respond to very diverse inputs (environmental cues) that activate various intracellular signaling pathways, and coordinately express necessary genes. It is thus conceivable that even to control the same gene, such as SM-MHC, SMC-subpopulations in different environments may need 10 to utilize distinct sets of regulatory pathways. In other words, the SM-MHC gene regulatory program evolved so that it utilizes various regulatory pathways to control transcription in heterogenous extra- and intracellular environments. In fact, the differential requirement of the intronic CArG and CArG2 of the SM-MHC gene supports a hypothesis that distinct transcriptional regulatory programs are activated in SMC-subtypes.

15 One striking feature of the intronic CArG mutant was that the transgene was completely silent in the elastic arteries such as the aorta, whereas expression was easily detectable in the intermediate and small size arteries directly branched from the aorta. There are at least two possible explanations for the differential requirement of the intronic CArG that are not necessarily mutually exclusive. First, we need to consider the heterogeneity in the embryonic origin of SMCs 20 between the large and smaller arteries. It has been postulated that SMCs have at least three embryonic origins: local mesenchymal cells, neural crest cells, and proepicardial cells. In the aorta, the aortic bulb and ascending aorta mainly consist of neural crest derived SMCs and the descending aorta mainly consists of mesenchymal derived SMCs. However, in intronic CArG mutant transgenic mice, SMCs in the aorta were stained negative irrespective of the position of the cells, 25 and no known difference in lineage fits the distribution of the intronic CArG-dependency. Therefore, it is unlikely that differences in embryonic origin solely determined the requirement of intronic CArG for transcriptional control.

In addition, as discussed above, the heterogeneity in phenotype and function of SMCs *in vivo* is likely to require multiple transcriptional programs to control the same gene. The 30 differences in the physiological functions of SMCs in elastic versus muscular arteries would also require SMCs to express distinct sets of genes to fulfill their functional roles. It is thus conceivable that the intronic CArG is integrated in a regulatory program that processes environmental cues unique to elastic arteries and controls gene expression important for the function of such vessels. A

number of genes including ion channels, contractile proteins, growth factors/receptors, and transcription factors have been shown to be differentially expressed in vascular beds. For example, a transcription factor, CHF1 (Hrt2/Hey2/HERP1/*gridlock*) has been shown to be mainly expressed in the aorta. It would be of interest to compare the transcriptional regulatory mechanisms of these genes, and also to determine the function of differentially expressed transcription factors in control of SMC-subtype-selective gene regulatory programs.

**Example 5: SRF Interaction with SM-MHC CArG Elements**

1. SMCs in intact tissues expressed SRF and other proteins that bind to CArG elements.

10 As an initial step to determine mechanisms that control SMC-subtype-specific transcriptional regulation through multiple CArG elements, we examined protein binding properties of each CArG element using EMSAs. Since there were no SMC culture cell lines that had been shown to faithfully emulate differentiated phenotypes of SMC-subtypes in terms of SM-MHC transcriptional control, we prepared nuclear extracts from intact rat tissues. As shown in Fig. 19, 15 each CArG probe formed several DNA-protein complexes with tissue nuclear extracts. The mobility of major shift bands (complexes A and B) formed with tissue nuclear extracts was the same as that with cultured SMC nuclear extracts. The mobility of complex A seen in tissues and culture cells was identical to that formed with recombinant serum response factor (SRF). Supershift assays using anti-SRF antibody showed that both complexes A and B contained SRF (Fig. 23). Several non-SRF shift bands that were specifically competed by cold self-competitors 20 but not by unrelated sequences (data not shown) were also formed in the EMSA experiment (complexes C-G). Each probe formed largely similar shift band patterns with the SM tissue nuclear extracts. However, the shift bands formed with liver or cultured SMC extracts were somewhat different from those formed with the SM tissues extracts. For example, CArG1, CArG2, and 25 intronic CArG probes formed complex E with liver and cultured SMC extracts, while this complex was not formed with the SM tissue extracts. Conversely, complex F formed the intronic CArG probe and SM tissue samples was not present in the liver samples. Although a further analysis is necessary to determine the significance of these non-SRF DNA-binding proteins in transcriptional regulation in cells, the data show that non-SRF DNA-binding proteins capable of binding to the 30 CArG probes may be differentially expressed in SM and non-SM tissues.

2. SRF binding of CArG elements of the SM-MHC gene within intact chromatin under physiological conditions

To directly address whether SRF bound the endogenous SM-MHC CArG elements, we employed chromatin immunoprecipitation (ChIP) assays. Intact cultured rat aortic SMCs, L6 rat myoblasts, L6 myotubes, and Rat1 fibroblasts were directly fixed with formaldehyde. Crosslinked chromatin was immunoprecipitated with anti-SRF antibody. The precipitated 5 chromatin DNA was then purified and subjected to PCR analysis for enrichment of the target sequences. The promoters of insulin,  $\beta$ -globin, and skeletal  $\alpha$ -actin genes (Fig. 24, Rows A-C), which are silent in SMCs, and a region (-4133 to -3832) of the SM-MHC 5'-flanking sequence (Fig. 24, Row D), which lacks CArG elements, were used in control reactions. Amplification of these sequences showed a background level of chromatin immunoprecipitation and PCR 10 amplification (Fig. 24, Rows A-D). However, anti-SRF antibody specifically enriched the 5'- flanking CArG region (CArG1 and CArG2) and the intronic CArG regions of the SM-MHC gene (Fig. 24, Rows E and F, lanes 3) in SMC chromatin as compared with the background amplifications of the promoters of negative control genes (Fig. 24, Rows A-D, lanes 3). Importantly, the same SM-MHC regions were not enriched in immunoprecipitation samples of L6 15 or Rat1 cells that do not express the SM-MHC gene (Fig. 24, Rows E and F, lanes 6, 9, 12). Since the promoter region of the skeletal  $\alpha$ -actin has been shown to contain CArG elements active in skeletal myocytes, this promoter was used as a positive control for L6 cells. As expected, the skeletal actin promoter was enriched in SRF immunoprecipitation samples from L6 myoblasts and myotubes (Fig. 24, Row C, lanes 6 and 9) but not in SMC or Rat1 cells, further demonstrating the 20 specificity of the SRF antibody in these experiments. It is important to note that the PCR detection methods used in these ChIP experiments are not quantitative. As such, it is impossible to determine the stoichiometry of SRF binding to the SM-MHC CArG elements. Nevertheless, the ChIP experiments indicated that at least some SM-MHC CArG regions were bound by SRF in chromatin in intact cultured SMCs. In addition, observations that SRF bound the CArG regions of 25 endogenous SM-MHC gene in chromatin in intact SMCs but not in L6 skeletal myocytes or Rat1 fibroblasts provide evidence that mechanisms exist *in vivo* to control SRF binding to the SM-MHC CArGs in a cells-specific manner.

### 3. Discussion

30 *SRF-dependent control of SM-MHC transcription in vivo:* The present studies provide evidence showing binding of SRF to the CArG elements of the endogenous SM-MHC gene in the context of intact chromatin as opposed to oligonucleotide fragments employed in typical

DNA binding studies. It has been shown that in an *in vitro* avian proepicardial cell differentiation system two types of dominant negative SRF inhibited SMC differentiation and reduced expression of SMC marker genes including SM  $\alpha$ -actin and SM22 $\alpha$ . These data demonstrate the significant role of SRF in the control of endogenous SMC differentiation marker genes. A critical question is 5 thus: How can SRF, which is not clearly SMC-specific, regulate SMC-specific gene expression? Various hypotheses have been postulated that are not mutually exclusive. First, although SRF is clearly not cell specific, there are very large differences in the level of SRF expression between different cell types that may contribute, at least in part, to cell specific SRF-CArG dependent gene expression. Second, the binding affinity of SRF may be regulated in a cell type dependent manner 10 by interactions with other proteins, such as MHOx, or by phosphorylation. Third, SRF may form SMC-specific multi-protein complexes. Although we did not observe SMC-specific higher order complexes in EMSA experiments, it is possible that a longer probe might form such a multi-protein complex in EMSAs. Lastly, chromatin remodeling may play a significant role in the regulation of 15 activity of transcriptional regulatory modules. It is now well established that transcription factor binding to *cis*-elements are greatly affected by chromatin structure. It has also been shown that various transcription factors bind histone acetylases and deacetylases and thereby modify chromatin structure.

The results of ChIP assays (Fig. 24) demonstrated that the SM-MHC CArG regions were bound by SRF only in SMCs, although nuclear extracts of L6 myocytes were perfectly 20 capable of binding the CArG elements in EMSAs (Manabe and Owens, unpublished observations). Conversely, SRF bound the skeletal  $\alpha$ -actin promoter only in L6 myocytes but not in SMCs or fibroblasts. These data are potentially extremely important in that they suggest that the transcriptional regulatory regions of the endogenous SM-MHC gene are only active in SMC 25 chromatin. That is, the transcriptional regulatory regions of the SM-MHC gene may be in "closed state" in the non-SM cell lines.

*SMC-subtype-selective transcription control in vascular diseases:* In contrast to the main function of mature SMCs (i.e., contraction), one of the major functions of SMCs in developing blood vessels is to contribute to formation of the vascular wall through cell proliferation and production of extracellular matrix components. Such functions are also extremely important 30 during repair of vascular injury, and may contribute to post-angioplasty restenosis. As such, it is likely that a part of the transcriptional regulatory programs that are normally activated in vascular development is re-activated by vascular injury and alters gene expression. It would be thus

important to study the functions of the CArG elements during vascular development and in neointimal formation induced by vascular injury.

It is well known that some vascular beds including the coronary arteries and aorta are more prone to atherosclerosis. Our data provide evidence for SMC-subtype-selective transcriptional regulatory mechanisms. It is tempting to speculate that this multiplicity in the transcriptional control mechanisms might in some way be related to differential susceptibility of different vessels to atherosclerosis. The modularity of the SM-MHC transcription program might also allow us to design gene therapy vectors to target specific subsets of SMCs. SMC-selective activity obtained by the intronic CArG region coupled with a minimal TK promoter in transgenic mice suggests that the region could be used as a building block for such vectors. The results of the present studies have revealed the complex nature of transcriptional control of the SM-MHC gene *in vivo* in SMC-subtypes, and the role that multiple *cis*-regulatory modules play in processing divergent environmental cues *in vivo*. Further studies on the SM-MHC gene regulation should provide additional insights into the complex and dynamic regulatory mechanisms that normally control SMC differentiation and how these processes are altered during phenotypic modulation of SMCs during injury repair and development of vascular diseases.

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Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention.